Mechanism of Poly(ADP-Ribose) Formation and the Role of PARP1 in Inflammation

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SUMMARY

Proteins are essential components of all living organisms and participate in virtually every cellular process. Many proteins are enzymes, which catalyze very specific biochemical reactions. Protein function and enzymatic activity are often regulated by so-called post-translational modifications. An important covalent protein modification is poly(ADP-ribosyl)ation (PARylation). PARylation is carried out by certain members of the protein family of poly(ADP-ribose) polymerases (PARPs), whose founding member is PARP1.

The aim of this thesis was to investigate the molecular mechanism of PARylation and to study the function of PARP1 for pro-inflammatory gene expression.

In order to study the molecular mechanism of poly(ADP-ribose) (PAR) formation, the enzymatic activities of the closely related PARP family members PARP1, PARP2 and PARP3 were characterized *in vitro*. By use of purified recombinant proteins and protein chimera, we found that the different protein domains of the three enzymes cooperate in a tight and very specific manner. While PARP1 and PARP2 catalyzed the formation of PAR, PARP3 only showed mono-ADP-ribosylation activity. We defined the enzymatic parameters K_m and V_{max} for the DNA dependent activation of PARP1 and found that lysine residues within an auto-modification loop of PARP1 are target sites for auto-ADP-ribosylation. Moreover, lysine residues in the amino-terminal basic tails of core histone molecules were identified as targets for trans-ADP-ribosylation by PARP1. Together, these results provide detailed insights into the enzymatic mechanism of PAR synthesis and challenge the traditional assumption, that PAR is attached onto glutamic acid residues.

To study the contribution of PARP1 for pro-inflammatory gene expression under the control of the transcription factor NF-κB, an *in vivo* model system for *Salmonella* induced colitis was employed. In this model, PARP1 was required for efficient expression of a subset of pro-inflammatory genes and loss of PARP1 caused a delayed inflammatory host response to *Salmonella* infection. Thus, PARP1 was identified as a novel host factor, which, due to its transcriptional co-activator function for pro-inflammatory gene expression, accelerates *Salmonella* induced colitis.

ZUSAMMENFASSUNG

Proteine sind als essentielle Bestandteile aller Lebewesen an praktisch jedem zellulären Prozess beteiligt. Viele Proteine sind Enzyme, die sehr spezifische biochemische Reaktionen katalysieren. Die Funktion von Proteinen und ihre enzymatische Aktivität werden häufig durch so genannte post-translationelle Modifikationen reguliert. Eine wichtige kovalente Proteinmodifikation ist die Poly(ADP-Ribosyl)ierung (PARylierung). Die PARylierung wird durch bestimmte Mitglieder der Proteinfamilie der Poly(ADP-Ribose) Polymerasen (PARPs) ausgeführt, deren Gründungsmitglied PARP1 ist.

Ziel dieser Arbeit war es, den molekularen Mechanismus der PARylierung sowie die Funktion von PARP1 bei der pro-inflammatorischen Genexpression zu untersuchen.

Um den molekularen Mechanismus der Synthese von Poly(ADP-Ribose) (PAR) genauer zu untersuchen, wurde die enzymatische Aktivität der eng verwandten PARP Familienmitglieder PARP1, PARP2 und PARP3 *in vitro* charakterisiert. Mit Hilfe von gereinigten, rekombinanten Proteinen und Proteinchimären ergab sich, dass die verschiedenen Proteindomänen der drei Enzyme sehr eng und spezifisch miteinander kooperieren. Während PARP1 und PARP2 die Synthese von PAR katalysierten, zeigte PARP3 lediglich Mono-ADP-Ribosylierungsaktivität. Wir bestimmten die enzymatischen Parameter K_m und V_{max} für die DNA-abhängige Aktivierung von PARP1 und erkannten, dass Lysinreste in einer Automodifikationsschleife von PARP1 als Ziel für die Auto-ADP-Ribosylierung fungieren. Darüber hinaus identifizierten wir Lysinreste in den amino-terminalen, basischen Histonausläufern als Zielaminosäuren von Trans-ADP-Ribosylierung durch PARP1. Zusammengenommen bieten diese Resultate einen detailierten Einblick in den enzymatischen Mechanismus der PAR-Synthese und hinterfragen die traditionelle Annahme, dass PAR an Glutamate angeheftet wird.

Um die Beteiligung von PARP1 bei der pro-inflammatorischen Genexpression unter der Kontrolle des Transkriptionsfaktors NF-κB zu untersuchen, wurde von einem *in vivo* Modellsystem für Salmonellen-induzierte Kolitis Gebrauch gemacht. In diesem Modell war PARP1 notwendig für die effiziente Expression von einer Untergruppe pro-inflammatorischer Gene und der Verlust von PARP1 führte zu einer

verzögerten Entzündungsreaktion nach Salmonelleninfektion. PARP1 wurde folglich als neuer Wirtsfaktor identifiziert, der wegen seiner transkriptionellen Ko-Aktivatorfunktion für pro-inflammatorische Genexpression beschleunigend wirkt auf eine Salmonellen-induzierte Kolitis.

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1 INTRODUCTION

The blueprint of life is stored in our genetic material, the DNA. DNA comprises protein-encoding regions, the genes, which are transcribed into a messenger called mRNA. mRNA in turn is translated into proteins, the executers of all main cellular functions and components of many cellular structures. Protein function is tightly controlled at several levels, one important being the level of post-translational modification. Post-translational modifications regulate enzyme activities, cellular localization, protein interactions and protein stability. Frequent protein modifications include phosphorylation, acetylation, methylation, ubiquitylation and sumoylation [1]. A fascinating post-translational modification in higher eukaryotes is poly(ADP-ribosyl)ation (PARylation), whereby short or large negatively charged chains of poly(ADP-ribose) (PAR) are generated from nicotinamide adenine dinucleotide (NAD^+) as a substrate and attached to target proteins [2]. This process is carried out by an enzyme family, which has been named PAR polymerases (PARPs). PARylation has been implicated in a multitude of cellular processes including the sensing and signaling of damaged DNA, the regulation of gene expression and the execution of different cell death programs [3]. Consequently, PARPs and PARylation play important and often diverse roles in carcinogenesis and tumor formation as well as in many inflammatory diseases [3-5].

1.1 Poly(ADP-ribosyl)ation

1.1.1 Discovery

PARylation was first discovered already more than 45 years ago by Pierre Chambon in Paul Mandel`s group [6]. In 1979, PARP1 was isolated and characterized as the protein mainly responsible for catalyzing the formation of PAR [7]. It was later found that PARP1 itself is the main acceptor protein for the attachment of PAR [8], although several other nuclear proteins including histones are also targeted by PARylation [9, 10].

1.1.2 Mechanism

During PARylation, NAD^+ is cleaved into ADP-ribose and nicotinamide. Multiple ADP-ribose moieties are connected via glycosidic ribose-ribose bonds to generate

linear and multiple branched chains of PAR of different length and complexity (Figure 1) [9, 11]. The PARylation reaction can be subdivided into three distinct steps: the initiation reaction, i.e. the attachment of the first ADP-ribosyl residue to an acceptor protein after NAD^+ cleavage; the elongation reaction, i.e. the addition of further ADP-ribose moieties onto already transferred ADP-ribose units; and the branching reaction, i.e. the branching off of ADP-ribose residues from a linear portion of the polymer to create a branching point (Figure 1) [9, 11, 12]. PAR chain length is heterogenous and was described to reach up to 200 ADP-ribose units *in vitro* [13]. Long homo-polymers of ADP-ribose are often irregularly branched with an average branching frequency of one branching point per 20 to 50 units of ADP-ribose [13].

Most PAR is generated *in vivo* within seconds to minutes after induction of oxidative stress and is subsequently quickly degraded by the different isoforms of PAR glycohydrolase (PARG) and/or by the ADP-ribose protein hydrolase 3 (ARH3) [14-17]. An ADP-ribosyl protein lyase has also been identified and implicated in the degradation of PAR [18]. The short half-life and rapid turnover of PAR suggest that PAR formation is a transient cellular response and that PAR levels are tightly controlled under physiological stress conditions.

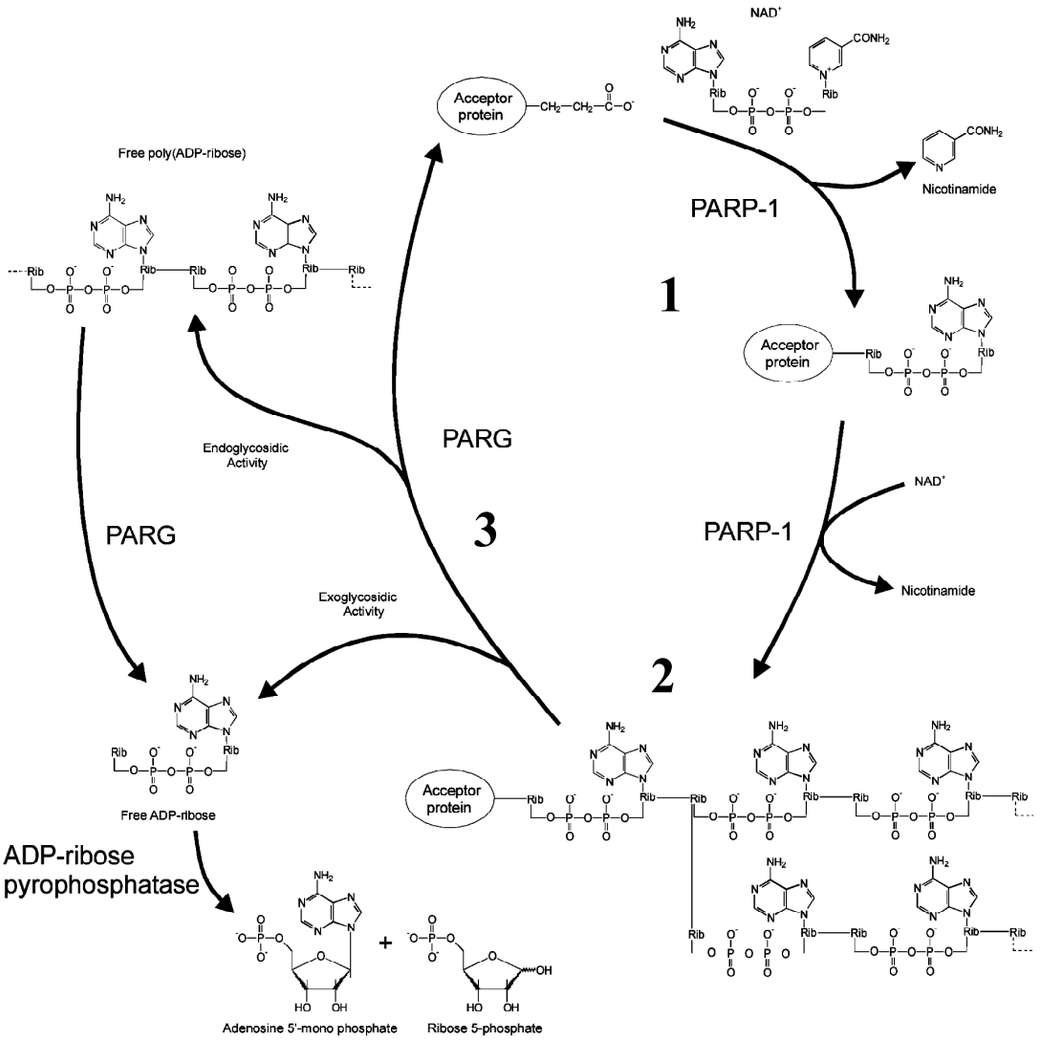

Figure 1: Metabolism of PAR formation. (1) In the initiation reaction, PARP1 cleaves the glycosidic bond between nicotinamide and ribose in NAD^+ to covalently modify acceptor proteins with an ADP-ribosyl unit. (2) PARP1 also catalyses the elongation and branching reaction, giving rise to polymers with chain lengths of up to 200 units and several branching points. (3) PARG catalyses the degradation of PAR to free ADP-ribose. Modified from [19].

1.1.3 Responsible enzymes

For many years, PARP1 was thought to be the only enzyme capable of catalyzing PAR formation. It was only when residual PAR levels were observed in PARP1 knockout mice in the mid nineties that additional PARP family members were subsequently discovered [20, 21]. In 1999, PARP2 was identified as an active PARP with high sequence homology to the catalytic domain of PARP1 [22, 23]. Up to now eighteen putative PARP family members have been proposed to exist in humans based on homology searches and *in silico* characterization [24, 25]. In mouse, rat and pufferfish, genes for 16 putative PARP members exist, while there are 12 PARP

orthologues in chicken, two in *Drosophila* and three in *C. elegans*. Also amoebae, slime molds and plants contain several PARPs [25]. No PARP orthologue has been identified in yeast, however. Many of the identified putative PARP family members in higher eukaryotes may not be active PAR polymerases, but instead either function as mono-ADP-ribosyl transferases or represent completely inactive proteins.

1.2 PARP family members

1.2.1 PARP1

Human PARP1 (EC 2.4.2.30) contains 1014 amino acids and has an apparent molecular weight of 113kDa. It is an abundant nuclear chromatin associated protein with approximately one million molecules per cell. PARP1 accounts for about 90 % of total PAR formation in human cells [21]. The enzyme has only low basal activity but is activated by double strand break mimicking DNA *in vitro* or by induction of DNA damage *in vivo* [26]. PARP1 and PAR formation were described to be involved in a variety of nuclear functions including DNA damage signaling or repair, modulation of chromatin structure and regulation of transcription [3].

PARP1 contains three functionally distinct domains: an amino-terminal DNA binding domain (DBD), a central auto-modification domain (AD), and a carboxyl-terminal PARP homology domain that includes the catalytic domain responsible for PAR formation (Figure 2) [27]. The DBD extends from the initiator methionine to threonine 373 in human PARP1. It contains two structurally and functionally unique zinc fingers (FI: aa 11-89; FII: aa 115-199) [28]. Recently, a third so far unrecognized zinc binding motif was discovered (FIII: aa 233-373) [29, 30]. This motif alone does not bind DNA but is essential for the catalytic activity of PARP1 [29]. The DBD also contains a bipartite nuclear localization signal (NLS) of the sequence KRK-X(11)-KKKSKK (aa 207-226) that targets PARP1 to the nucleus [31]. The PARP1 zinc fingers FI and FII are thought to recognize altered structures in DNA rather than particular sequences and have also been reported to be involved in protein–protein interactions [32]. PARP1 strongly associates with DNA single and double strand breaks generated either directly by DNA damage or indirectly by the enzymatic excision of damaged bases during DNA repair processes [28]. Several studies indicate that the first zinc finger is required for PARP1 activation by both DNA single and double strand breaks, whereas the second zinc finger may exclusively act as a DNA

single strand break sensor [28]. More recently, PARP1 was shown to bind to and become activated by intramolecular DNA quadruplexes [33].

The auto-modification domain of PARP1 is located in the central region of the enzyme, between residues 373 and 525 of human PARP1 [34, 35]. It was identified as the domain containing acceptor amino acids for the covalent attachment of PAR [36]. Multiple acceptor sites for PAR were suggested to exist within PARP1 but no individual site has yet been identified by site-directed mutagenesis or mass spectrometry. The AD of PARP1 comprises a breast cancer 1 protein (BRCA1) C-terminus (BRCT) domain (from aa 386 to 464 in human PARP1) as well as an unstructured loop that connects the AD with the PARP homology domain. Besides auto-modification, PARP1 is subject to a number of additional post-translational modifications: lysine residues within the AD of PARP1 can be acetylated by the histone acetyltransferase (HAT) p300/CBP [37]. In proteomic approaches PARP1 was also found to be a target for covalent lysine sumoylation and phosphorylation [38-41].

PARP1 contains an 80-90 amino acid long WGR domain carboxyl-terminal of the AD. The WGR domain is named after the most conserved central motif of tryptophane (W), glycine (G), arginine (R) residues and may represent a nucleic acid binding domain [28]. This region of PARP1 has not been extensively characterized and its function is still unknown.

The catalytic domain has been suggested to catalyze at least three different enzymatic reactions: the attachment of the first ADP-ribose moiety onto an acceptor amino acid (initiation reaction), the addition of further ADP-ribose units onto already existing ones (elongation reaction), and the generation of branching points (branching reaction) [27]. The active site is formed by a phylogenetically well-conserved sequence of approximately 50 residues (aa 859–908 of hPARP1). This 'PARP signature' contains the NAD^+ acceptor sites and critical residues involved in the initiation, elongation and branching of PAR. The catalytic glutamate at position 988 in human PARP1 seems to be of special importance, since this residue is essential for PAR chain elongation but not for the NADase activity, i.e. the hydrolysis of NAD^+ to ADP-ribose and nicotinamide [3].

Despite intensive research over the last decades, the molecular mechanism of PAR formation has not been comprehensively understood. In particular, unresolved issues are how DNA binding in the amino-terminal DBD triggers enzyme activation

in the carboxyl-terminal catalytic domain and which amino acids are targeted by the auto-modification reaction.

1.2.2 PARP2

The 65kDa protein PARP2 is the PARP family member that most closely resembles PARP1 with 60% amino acid identity between these two proteins in the PARP homology domain [42]. PARP2 is less active than PARP1 but is believed to be responsible for most of the residual PAR formation activity in PARP1 deficient cells [21, 22]. In response to DNA damage, PARP2 was estimated to contribute 5% to 10% of the total PAR synthesis in PARP1/PARP2 proficient cells [35]. Just like PARP1, PARP2 is found exclusively in the cell nucleus. It has been postulated previously that a non-conventional bipartite NLS of the sequence (KK_{20}-X_{15}-$KKMRTCQRK_{44}$) in the amino-terminal region of the protein is responsible for nuclear import [22, 43]. This bipartite NLS was identified based on amino acid sequence analysis but was not experimentally investigated in the context of full-length PARP2. Moreover, the mechanism of PARP2 nuclear translocation has not been elucidated [43].

PARP2 contains a WGR domain but lacks most motifs present in the amino-terminal half of PARP1 (Figure 2). Neither a zinc binding motif nor a BRCT domain has been described for PARP2. In contrast to PARP1, PARP2 contains an amino-terminal basic SAP/SAF motif, a eukaryotic module proposed to be involved in sequence- or structure-specific DNA and RNA binding [44]. In line with the fact that PARP1 and PARP2 contain different DNA binding modules, the two proteins seem to have different binding specificities for certain DNA structures. Consequently their functions may be complementary but not completely overlapping. Functional redundancy (at least during early embryonic development) is, however, suggested by the finding that PARP1/PARP2 double knockout mice die at the onset of gastrulation, whereas both single knockouts are viable [45]. Unique functions of PARP2 on the other hand include spermatogenesis, adipogenesis and T cell development [44].

1.2.3 PARP3

PARP3 is the smallest PARP identified so far with two splice variants containing 540 and 533 amino acids, respectively [46]. The protein domain structure of PARP3 is very similar to that of PARP2, featuring a small putative DBD consisting of only 54 residues and apparently containing an amino-terminal targeting motif that is sufficient

to localize the long splice variant to the centrosome [46, 47]. PARP3 expression was recently described to be tightly regulated and restricted to only specific cell types and tissues [48]. The enzymatic properties of PARP3 are only poorly investigated and it is currently still a matter of debate whether PARP3 possesses PARylation activity or merely catalyzes mono-ADP-ribosylation.

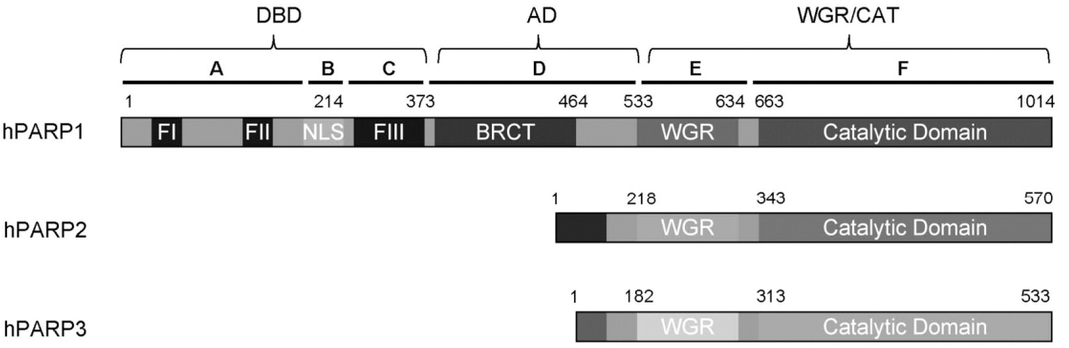

Figure 2: Domain organization of human PARP1, PARP2 and PARP3. Letters A-F indicate domain nomenclature of PARP1, numbers indicate amino acid positions. AD, auto-modification domain; BRCT, breast cancer 1 protein (BRCA1) C-terminus domain; CAT, catalytic domain; DBD, DNA binding domain; FI, zinc finger I; FII, zinc finger II; FIII, zinc binding motif III; NLS, nuclear localization signal; WGR, tryptophane, glycine, arginine rich domain.

1.2.4 PARP structures

Attempts to obtain structural information on the full-length proteins PARP1, PARP2 and PARP3 by x-ray crystallography or NMR have not been successful up to now. The three-dimensional structures of single domains, however, have been solved and allow structure-based comparisons of different PARP family members (Figure 3) [27, 49] (PDB: 1A26, 1GS0, and 2PA9). Although the amino acid identity between PARP1 and PARP2 or PARP3 is only moderate, the overall structure of the catalytic domains of these three proteins is nearly identical. This conservation suggests, in general, similar capabilities to generate PAR. Both PARP1 and PARP2 have been shown to synthesize very complex branched polymers at least *in vitro* [28].

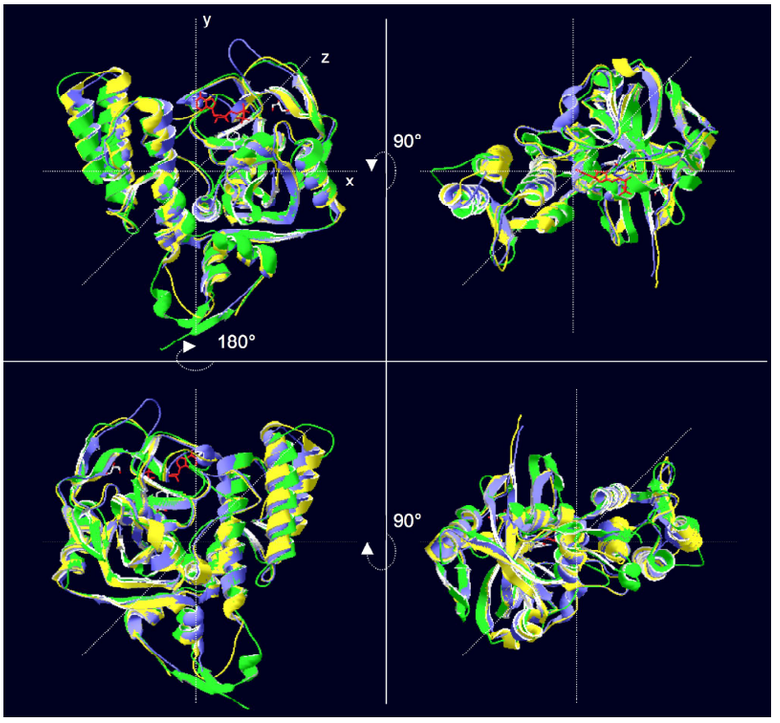

Figure 3: Overlay of the crystal structures available for the catalytic domains of chicken PARP1 (PDB: 1A26), mouse PARP2 (PDB: 1GS0) and human PARP3 (PDB: 2PA9). PDB files were obtained from RCSB PDB (www.rcsb.org/pdb/home/home.do), the alignment was performed using the Magic Fit function of the Swiss-PDBViewer with the catalytic domain of chicken PARP1 as template. Yellow: PARP1, blue: PARP2, green: PARP3.

1.2.5 Other PARP family members

Other PARP family members include cytoplasmic vault PARP (PARP4, vPARP) [50] and the telomeric tankyrases 1 and 2 (PARP5a and PARP5b) [42, 51] as well as several proteins with homology to PARP1 but without described PARylation activity. Accordingly, these proteins were proposed to not be considered true PARP family members but instead be renamed PARP-like mono-ADP-ribosyl-transferases (Pl-MARTs) [28].

1.3 Role of PARP1 in chromatin remodeling

Chromatin refers to the protein bound state of DNA within the cell nucleus. The nucleosome is the simplest unit of chromatin and is composed of an octamer of the four core histones H2A, H2B, H3 and H4 around which 147 base pairs of DNA are wrapped [52]. The histone octamer is predominantly globular except for the amino-terminal unstructured histone tails. Especially the tails possess a large number of modified residues with distinct types of modifications such as acetylation,

methylation, phosphorylation, ubiquitylation, sumoylation, proline isomerization and deimination [52]. Many of these modifications influence chromatin structure and therefore DNA dependent processes such as DNA replication, repair and transcription.

A relationship between PARP1, PARylation (potentially DNA damage independent) and chromatin organization has been proposed and studied *in vitro* already more than 30 years ago [53-55]. PARylation of all histones was early demonstrated [53, 56, 57] and decompaction of chromatin structure by histone PARylation was observed soon later [58, 59]. Besides covalent modification of histones with PAR, histones and many chromatin associated non-histone proteins also interact non-covalently with PAR *in vitro* [60-63]. Whereas PARP1 was suggested to displace H1 from chromatin at least in the MCF-7 breast cancer cell line [64], ISWI was shown to promote the association of H1 with chromatin [65].

PARP1 may exert its functions on chromatin structure modulation also by additional ways. For instance, the activity of the ATP-dependent chromatin remodeler ISWI was shown to be regulated by PARP1 *in vitro* and *in vivo* [66]. PARylation of ISWI inhibited its ATPase activity by reducing the affinity of ISWI for nucleosomes. PARP1 and ISWI may act antagonistically in the regulation of chromatin structure by antagonistically regulating the association of linker histone H1 with chromatin. The macrodomain containing oncogene ALC1, a member of the SNF2 ATPase superfamiliy, was recently shown to interact with PAR by two independent research groups [67, 68]. ALC1 is recruited to sites of PAR formation in cells and its ATP-dependent chromatin remodeling activity is stimulated by PARylated PARP1. Another interesting target for PARylation by PARP1 is the histone exchange factor FACT (facilitates chromatin transcription) [69]. ADP-ribosylation of FACT induces its dissociation from the nucleosome and thereby inhibits FACT mediated exchange of histone variant H2AX. Heterochromatin formation may also be regulated by PARylation, because the heterochromatin protein 1 α (HP1α) was shown to be modified by both PARP1 and PARP2 with PARylation affecting HP1α binding to chromatin [70].

PAR is recognized and bound by macrodomain containing histone variants, which may regulate chromatin structure and function [71]. Very recently, macroH2A1.1 was shown to specifically recruit to sites of PARP1 activation and PAR

formation after laser-induced DNA damage and to transiently compact chromatin in a PAR-dependent manner [72]. Following this report, macrodomains have been extensively discussed as novel PAR binding modules *in vivo* with roles in chromatin structure modulation, DNA damage detection and repair [73, 74].

Moreover, PARP1 has been shown to regulate the function of the insulator protein CTCF [75]. Insulators are DNA elements, which help to organize the genome into regulatory units by preventing the spread of heterochromatin and limiting the effects of enhancers [76]. Guastafierro et al. reported that CTCF stimulates DNA damage independent PAR formation *in vitro* and is PARylated *in vivo,* which in turn affects its insulator function [77].

Auto-modified PARP1 was recently shown to localize to the DNMT1 promotor and protect it from methylation [78]. Furthermore, PARP inhibition induced global DNA hypermethylation, suggesting that PAR formation counteracts DNA methylation [79].

In summary, research that has started already in the 1970s together with more recent findings of the last decade strongly support the notion of PARylation being an essential chromatin mark with important functions in many aspects of chromatin structure regulation.

1.4 PARP1 and DNA damage

Because PARP1 binds to and is activated by damaged DNA, a role in DNA damage sensing, signaling and repair has been proposed [80-86]. PARP1 is efficiently activated by genotoxic stress induced by a variety of stimuli, such as reactive oxygen species (e.g. H_2O_2 treatment), irradiation-induced DNA strand breaks, or alkylating agents [84]. Furthermore, PARP1 was shown to interact with a number of proteins involved in DNA damage repair pathways, including XRCC1 [87, 88], DNA ligase IIIα [89], DNA polymerase β [90], Ku70/80 [91], TopBP1 [92] and PCNA [93]. Many of these proteins also bind to PAR at least *in vitro* [62, 63] and were discussed to be direct targets for covalent PARylation [9, 24, 94].

Using laser microirradiation in single living cells, Haince et al. showed that PARP1 very rapidly recruits to DNA strand breaks and that the fast accumulation of the repair proteins MRE11 and NBS1 at damage sites depends on PARP1 [95]. Of note, PARP1 accumulation after laser microirradiation was observed even before γ-

H2AX foci appeared or ATM and 53BP1 were recruited [95]. Another similar study established that PARP1 is recruited to sites of laser microirradiation within seconds but then begins to dissociate from the damage sites after only approximately 1 minute and that this dissociation depends on the enzymatic activity of PARP1 [96].

Together, a plethora of data generated during the last decades suggests a role for PARP1 and PARylation in different DNA repair pathways. How important these roles are, however, is difficult to estimate. First, there is conflicting literature regarding the role of PARP1 in DNA repair pathways and several studies have failed to show differences in DNA repair between wild type and PARP1 deficient cells [97-99]. Second, PARP1 deficient mice are viable, fertile, have a normal lifespan and do not develop spontaneous tumors [100]. Thus, PARP1 may not be an essential DNA repair enzyme in the stricted sense, but may aid the cellular DNA repair machinery under certain stress conditions [101].

1.5 PARP1 and transcription

Chromatin immunoprecipitation coupled to hybridization to genome microarrays (ChIP-chip) has shown that PARP1 binding is enriched at the promoters of many RNA polymerase II transcribed genes [64]. In gene expression microarray analysis performed with embryonic stem cells and livers from PARP1 knockout and wild type control mice, about 3.5% of the transcriptome under non-stimulated conditions was regulated by PARP1 with about 60-70% of the PARP1 dependent genes being positively regulated [102].

The mechanism best investigated today by which PARP1 is regulating gene expression is the promoter specific co-regulator function (either as co-activator or co-repressor) for a number of transcription factors. One of the first transcription factor families, which was described to be regulated by PARP1, is a family of inducible transcription factors called nuclear factor-kappaB (NF-κB), which induces the expression of many pro-inflammatory genes, such as cytokines, chemokines, adhesion molecules and inflammatory mediators [103]. The NF-kB family consists of five members, p50, p52, p65 (RelA), c-Rel and RelB [104]. All five proteins share an amino-terminal Rel homology domain (RHD) responsible for DNA binding and homo- and heterodimerization. NF-κB dimers bind to κB sites in the promoter/enhancer regions of target genes to regulate their transcription. Co-

activators and co-repressors are commonly recruited together with NF-κB to regulate target gene expression [104]. The strongest indication for a direct role of PARP1 in NF-κB-dependent transcription is the impaired expression of NF-κB-dependent pro-inflammatory mediators in PARP1 knockout mice [100, 105-107]. Mechanistic studies revealed that PARP1 is not required for the nuclear translocation of NF-κB, but that PARP1 directly interacts with both subunits of NF-κB (p65 and p50) and functions as a co-activator for NF-κB driven gene expression, a function independent of the DNA binding function of PARP1 and independent of PARylation activity [108, 109]. A role of PARP1 in transcriptional regulation of NF-κB dependent gene expression is now widely accepted. There is currently no consensus in the literature, however, concerning the role of PARylation for NF-κB regulated gene expression. Whereas initial studies, which included overexpressed catalytically inactive PARP1 mutants, showed that the physical presence of the protein but not its enzymatic activity was required for NF-κB driven reporter gene expression [108], other studies including PARP inhibitors suggested later that PARylation does have an impact on NF-κB mediated gene expression [110]. As PARP inhibitors may also target cellular proteins other than PARP1, these studies should be interpreted cautiously. Taking this into account, the precise role of PARP1 activity for NF-κB target gene expression remains still to be defined and may well depend on cell type, cell cycle stage, the stimulus used and the gene locus investigated.

More recently, PARP1 was also described to regulate the nuclear factor of activated T cells (NFAT) family of transcription factors [111, 112]. How PARP1 exactly influences NFAT function and whether the enzymatic activity of PARP1 is required for the regulation of NFAT remains unclear in these two partly contradictory reports. In 2009, the forkhead box O transcription factor FOXO1 was described to be negatively regulated by PARP1 [113]. Notably, the co-repressor function of PARP1 at FOXO1 regulated genes like $p27^{Kip1}$ was independent of PARylation activity, despite the fact that FOXO1 was modified by PARP1 and the functional significance of FOXO1 PARylation remains unaddressed. PARP1 also interacts with and functions as transcriptional co-regulator for nuclear respiratory factor 1 (NRF-1) independent of PARP1 enzymatic activity [114]. Moreover, while the transcription factors activator protein-1 (AP-1), AP-2, octamer-binding transcription factor-1 (Oct-1), yin-yang-1 (YY-1), transcription enhancer factor-1 (TEF-1), B-MYB, and TCF-4/β-catenin have

all been shown to only bind PARP1, p53 and fos were reported to bind PARP1 and to become PARylated [115-122]. PARP1 is furthermore required for the expression of the transcription factors Sp1 and NFI [123]. Sp1 was shown to be PARylated in response to H_2O_2 treatment and Sp1 PARylation correlated with a reduction of Sp1 DNA binding.

W.L. Kraus and his group recently published that PARP1 and the linker histone H1 reciprocally bind to RNA polymerase II transcribed promoters with PARP1 being enriched and H1 levels being reduced at actively transcribed genes [64]. PARP1 bound around the transcription start site (TSS) and PARP1 knockdown let to H1 binding to normally PARP1 occupied promoters and gene repression. The authors concluded that PARP1 functions to displace H1 from regions around the TSS to enhance transcription. In a follow-up publication, Kraus and colleagues reported that many PARP1 dependent genes are also regulated by PARG [124]. PARP1 and PARG were present at the promotor regions of the same genes and seemed to act in concert. Interestingly, the enzymatic activity of both proteins was often not required for target gene expression. Of note, Kraus` group analyzed transcript levels under basal non-stimulated conditions only. How PARP1 and PARG regulate gene expression of inducible genes upon stimulation was not analyzed and remains unresolved.

Together, PARP1 exerts its functions on gene expression via different mechanisms including transcriptional co-regulator function, regulation of insulator function, regulation of DNA methylation, displacement of histones and various effects on global or locus specific chromatin structure [125].

1.6 PARP1 and cell death

In the 1980s it was first proposed that over-activation of PARP1 in response to strong DNA damage can lead to cell death associated with depleted cellular energy pools [126, 127]. It was noted earlier, that strong PARP1 activity results in the rapid consumption of NAD^+ and consequently in the almost complete depletion of cellular NAD^+ pools [128, 129]. It was suggested that NAD^+ depletion in turn would lead to subsequent ATP depletion in an attempt of the cell to re-synthesize NAD^+ at the cost of ATP. Eventually, this energy crisis would cause necrotic cell death according to the "PARP suicide" model [127].

A second mechanism for PARP1 dependent cell death relates to the finding that PARP1 activation correlates with and may be required for the release of apoptosis

inducing factor (AIF) from the mitochondria [130]. Upon release from the mitochondrial inter-membrane space, AIF translocates to the cell nucleus and induces caspase-dependent or -independent apoptosis [131]. How exactly PARP1 activation and PAR formation inside the nucleus trigger AIF release from the mitochondria is, however, not clear at the moment and only the elucidation of the involved signaling mechanisms will provide formal proof to directly link PAR synthesis to AIF release. PARP1, PAR and AIF may function together as a sensory system to integrate information from the nucleus and the mitochondria on the genomic and metabolic state of a cell to mediate death/survival pathways [28].

In summary, two mechanisms of PARP1 and PAR dependent cell death have thus been defined, one leading to necrosis and involving NAD^+ and ATP depletion, another leading to apoptosis via AIF release [132].

1.7 PARP1 and inflammation

Inflammation occurs as a defensive response to a pathogenic insult in an attempt to remove the insult while limiting tissue damage [133]. Inflammation can, however, be very harmful and in fact is an important cause of morbidity and mortality in humans. Inflammation is associated with immediate infiltration of immune cells, secretion of cytokines and release of nitric oxide and reactive oxygen species to combat a pathogen. PARP1 is activated by intracellular oxidative stress, can mediate necrotic and apoptotic cell death and is involved in pro-inflammatory gene expression. Mice lacking PARP1 are viable and are protected from tissue injury in various oxidative stress-related disease models ranging from stroke, (MPTP)-induced parkinsonism, myocardial infarction, streptozotocin-induced diabetes, lipopolysaccharide-induced septic shock, arthritis, to colitis and zymosan-induced multiple organ failure [20, 28, 100]. These findings suggest an important role of PARP1 during inflammation. The protective effects observed in PARP1 knockout animals correlate well with the reduced expression of pro-inflammatory cytokines under the control of NF-κB, indicating that the role of PARP1 as co-activator for NF-κB target gene expression may be the most important underlying mechanism [3, 100, 105-107].

1.8 PARP1 and aging - at the crossroad of metabolism and inflammation

Aging is a multi-factorial process defined as time-dependent general decline in physiological function, which is associated with a progressively increasing risk of

morbidity and mortality [134, 135]. Age-associated diseases have complex etiology and underlying pathogenic mechanisms. Intensive efforts have been made over the last decades to identify single key players at the molecular level, which are involved in age-related diseases. PARP1 functions as molecular stress sensor and as such is involved in the cellular response to a variety of age-associated stress signals. The enzymatic activity of PARP1 is activated by reactive oxygen species (ROS), the levels of which are believed to increase with age and also drive the aging process. PARylation requires NAD^+ consumption and thus is directly linked to energy metabolism. Moreover, PARP1 shares its substrate molecule NAD^+ with other enzymes involved in aging, the most prominent being the family of NAD^+-dependent class III histone deacetylases (SIRTs). As transcriptional coactivator for NF-κB-dependent gene expression, PARP1 is connected to the immune response, which is implicated in almost all age-related diseases. Furthermore, numerous experimental studies have demonstrated the beneficial effects of PARP inhibition for several age-associated diseases. Together, this led us propose PARP1 to be a central key player during aging as an integrator at the crossroad of metabolic stress and inflammation [136].

1.9 PARP inhibition and inflammatory diseases

The first non-selective broad-range inhibitor of PARP activity, 3-aminobenzamide, was discovered more than 25 years ago and during the last two decades over 50 potential PARP inhibitors have been developed [101]. The involvement of PARP1 in cell death and the capacity of PARP1 to promote the transcription of pro-inflammatory genes are particularly important for the development of drugs, which target pathophysiological situations of detrimental inflammation. On the basis of structural information available for the catalytic domains of PARP1 and PARP2 co-crystallized with NAD^+ or certain PARP inhibitors, it became clear that the majority of PARP inhibitors mimic the nicotinamide moiety of NAD^+ and bind to the donor site within the catalytic domain [27, 49, 137]. Although the physiological functions of PARPs and PARylation is still under debate, numerous experimental studies during the last years have clearly demonstrated the beneficial effects of PARP inhibition from cell culture systems to pre-clinical animal models of acute and chronic inflammation [101, 138]. In general, the severity of many inflammatory diseases is suppressed by PARP inhibitors and the production of multiple pro-inflammatory

mediators is downregulated [139]. The reduced expression of pro-inflammatory genes by PARP inhibition is in contradiction to the finding that the enzymatic activity of PARP1 is not required for NF-κB dependent gene expression [108]. However, this discrepancy might be explained in three ways: First, it should be noted that the currently available PARP inhibitors do not discriminate well between PARP1 and other PARP family members or even other NAD^+-metabolizing enzymes, which are described to also play a role in inflammatory response pathways [140, 141]. Second, based on recent reports, one cannot exclude the possibility that PARP inhibitors might even affect non-NAD^+-consuming targets such as AKT/PKB or MMPs [142]. Third, the enzymatic activity of PARP1 might be required for the transcriptional activity of transcription factors involved in inflammatory processes other than NF-κB. Several groups have shown that co-operative activities between transcription factors such as AP-1, STAT-1 or IRF-1 in the enhanceosomes of NF-κB dependent genes are required for full synergistic activation of target genes [143, 144]. Considering these constraints of all currently available PARP inhibitors, the specific contribution of PARP1 enzymatic activity for inflammatory diseases, in which PARP inhibition has beneficial effects, needs to be evaluated very carefully.

1.10 PARP inhibition in cancer treatment

Many of the agents used in cancer treatment exert their cytotoxic effects by causing DNA damage. Conventional cytotoxic therapies, such as ionizing radiation or chemotherapy, are clinically beneficial because tumors deal less efficiently with DNA damage than normal tissue [145]. This is especially true for tumors with defects in the cellular repair systems responsible for faithful repair of damaged DNA. Consequently, the rational for the use of DNA repair inhibitors in cancer therapy relies on the assumption that inhibition of defined repair pathways will increase the likelihood that DNA damage will cause cell death specifically in tumor cells [145].

The breast cancer susceptibility proteins BRCA1 and BRCA2 function in the homologous recombination DNA repair pathway and loss of function of either protein is associated with an increased risk of developing cancer [145]. In fact, mutations in BRCA1 or BRCA2 account for 80-90% of all hereditary breast cancers [146]. In these cells, which are impaired in homologous recombination DNA repair, backup repair pathways may operate and deal with damage normally repaired via BRCA1/2

dependent pathways. Deficiency in one DNA damage repair component would thus render tumor cells highly sensitive to inhibition of a backup pathway, a concept called "synthetic lethality" [145]. Thus, inhibition of DNA damage signaling or repair might enhance the cytotoxicity of DNA damaging agents in a tumor specific manner. Since PARP1 and also PARP2 activity have been implicated in the sensing, signaling and repair of DNA damage, PARP inhibition has become an interesting option to treat cancers with defects in certain DNA repair pathways, especially in homologous recombination. Interestingly, cells defective for BRCA1 and BRCA2 are profoundly sensitive to PARP inhibition [147, 148] and chemical PARP inhibitors have been used in clinical trials with minimal toxicity to treat BRCA1- and BRCA2 deficient ovarian cancers [145]. In agreement with the concept of synthetic lethality, PARP inhibitors enhance the cytotoxic effects of a variety of agents commonly used in cancer treatment, such as alkylating agents (e.g. temozolomide), topoisomerase I inhibitors (e.g. irinotecan), and ionizing radiation. Recently, Mendes-Pereira et al. showed that also cells with mutations in the tumor suppressor gene phosphatase and tensin homolog (PTEN) are sensitive to PARP inhibition [149]. PTEN is involved in homologous recombination and is one of the most commonly mutated genes in human cancers. Thus, the data by Mendes-Pereira and colleagues suggest that the clinical assessment of PARP inhibitors should not be restricted to patients with BRCA1/2 mutations but be extended to a larger group of patients with mutations in PTEN.

Although PARP inhibition at the moment seems very promising for cancer treatment in combination with other cytotoxic anti-cancer drugs, the molecular mechanisms underlying the observed beneficial effects in patients in clinical trials are far from being understood. In particular, it remains to be experimentally established whether PARP inhibition in fact increases cytotoxicity exclusively because DNA damage repair is impaired or whether other, as yet undefined mechanisms are responsible for PARP inhibition dependent cell death. Moreover, as all PARP inhibitors used today belong to a class of NAD^+ analoga and target the NAD^+ binding pocket within the catalytic domain of PARPs, inhibition of other NAD^+-binding proteins has to be expected and should be carefully investigated. Finally, as promising as the clinical trials with PARP inhibitors are, the long-term effects of PARP inhibition in humans have not been studied and it remains to be shown that long-term inhibition of a protein postulated to be involved in DNA repair has no or neglectable adverse effects.

1.11 Aim of this thesis

The nuclear protein PARP1 plays important roles in DNA damage signaling, chromatin organization, regulation of gene expression and cell death. These functions may depend on or may be independent of the enzymatic activity of PARP1, i.e. the generation of PAR. The aim of this thesis was to investigate the molecular mechanism of PAR formation and the role of PARP1 for inflammation-induced gene expression.

2 RESULTS

2.1 Published review articles in peer reviewed journals

2.1.1 Poly(ADP-ribose) polymerase 1 at the crossroad of metabolic stress and inflammation in aging

Authors: Matthias Altmeyer and Michael O. Hottiger

Journal: Aging. 2009 May;1(5):458-469.

Contribution: Literature research; preparation of the model; drafting of the manuscript.

2.2 Published research articles in peer reviewed journals

2.2.1 Molecular mechanism of poly(ADP-ribosyl)ation by PARP1 and identification of lysine residues as ADP-ribose acceptor sites

Authors: Matthias Altmeyer, Simon Messner, Paul O. Hassa, Monika Fey and Michael O. Hottiger

Journal: Nucleic Acids Res. 2009 Jun;37(11):3723-38.

Contribution: Planning and designing the study; planning, performing and evaluating all experiments shown in Figures 1-6 and Supplementary Figures 1-6; in particular cloning, expression and purification of wild-type proteins, protein chimera and mutant proteins; *in vitro* mono- and poly(ADP-ribosyl)ation assays; time-course experiments; evaluation of enzyme kinetics; interaction studies; structural modeling and sequence alignments; preparation of all figures and drafting of the manuscript.

2.2.2 Identification of lysines 36 and 37 of PARP-2 as targets for acetylation and auto-ADP-ribosylation

Authors: Sandra S. Haenni*, Paul O. Hassa*, Matthias Altmeyer*, Monika Fey, Ralph Imhof and Michael O. Hottiger
* equal contribution

Journal: Int J Biochem Cell Biol. 2008;40(10):2274-83.

Contribution: Planning, performing and evaluating experiments; in particular interactions studies between PARP2 and the histone acetlytransferase (HAT) GCN5 (Figure 1E); *in vitro* acetylation assays (Figure 2D, Suppl. Figure 1); PARP2 expression analyses (Suppl. Figure 3); determination of PARP2 and HAT localization by immunofluorescence (Suppl. Figure 5); preparation of figures and revision of the manuscript.

2.2.3 Importin alpha binding and nuclear localization of PARP-2 is dependent on lysine 36, which is located within a predicted classical NLS

Authors: Sandra S. Haenni*, Matthias Altmeyer*, Paul O. Hassa*, Taras Valovka, Monika Fey and Michael O. Hottiger
* equal contribution

Journal: BMC Cell Biol. 2008 Jul 21;9:39.

Contribution: Planning, performing and evaluating experiments; in particular PARP2 localization studies with single amino acid mutants (Figure 4); interaction analysis (Figure 5D); sequence alignments (Figure 1A); preparation of figures and revision of the manuscript.

2.2.4 Sumoylation of poly(ADP-ribose) polymerase 1 inhibits its acetylation and restrains transcriptional coactivator function

Authors: Simon Messner, David Schuermann, Matthias Altmeyer, Ingrid Kassner, Darja Schmidt, Primo Schär, Stefan Müller and Michael O. Hottiger

Journal: FASEB J. 2009 Jul 31.

Contribution: Analysis of purified poly(ADP-ribose) generated by sumoylated or desumoylated PARP1 using DNA sequencing gel electrophoresis and silver staining (Figure 3C).

Only the title page of this article is presented in the following section of this thesis. The complete article can be obtained from the journal homepage.

2.2.5 A macrodomain-containing histone rearranges chromatin upon sensing PARP1 activation

Authors: Gyula Timinszky*, Susanne Till*, Paul O. Hassa*, Michael Hothorn, Georg Kustatscher, Bianca Nijmeijer, Julien Colombelli, Matthias Altmeyer, Ernst H. K. Stelzer, Klaus Scheffzek, Michael O. Hottiger and Andreas G. Ladurner

* equal contribution

Journal: Nat Struct Mol Biol. 2009 Sep;16(9):923-9.

Contribution: Cloning, expression and purification of recombinant human PARP1 and PARP2; preparation of AGS shMock and shPARP1 cells; analysis of PARP1 knock-down efficiency.

Only the title page of this article is presented in the following section of this thesis. The complete article can be obtained from the journal homepage.

2.3 Submitted research articles to peer reviewed journals

2.3.1 PARP1 accelerates *Salmonella* induced gut inflammation

Authors: Matthias Altmeyer, Manja Barthel, Matthias Eberhard, Hubert Rehrauer, Wolf-Dietrich Hardt and Michael O. Hottiger

Journal: Submitted to Gastroenterology

Contribution: Performing and evaluating experiments; in particular analysis of whole genome expression arrays (Figures 1C, 4A, 4B, 4C, 4D), qRT-PCR (Figures 1D, 2A, 3F, 5A, 5B, 5C, Suppl. Figures 1A, 2), Western blot (Figure 2B), immuofluorescence in tissue sections and cultured cells (Figure 2C, Suppl. Figures 1B, 1C); statistical analyses; preparation of the figures and tables; writing of the manuscript.

Poly(ADP-ribose) polymerase 1 at the crossroad of metabolic stress and inflammation in aging

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Key words: *PARP-1, NAD+, ROS, NF-κB, inflammation, aging*
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Abstract: Poly(ADP-ribose) polymerase 1 (PARP1) is a chromatin-associated nuclear protein, which functions as molecular stress sensor. Reactive oxygen species, responsible for the most plausible and currently acceptable global mechanism to explain the aging process, strongly activate the enzymatic activity of PARP1 and the formation of poly(ADP-ribose) (PAR) from NAD^+. Consumption of NAD^+ links PARP1 to energy metabolism and to a large number of NAD^+-dependent enzymes, such as the sirtuins. As transcriptional cofactor for NF-κB-dependent gene expression, PARP1 is also connected to the immune response, which is implicated in almost all age-related or associated diseases. Accordingly, numerous experimental studies have demonstrated the beneficial effects of PARP inhibition for several age-related diseases. This review summarizes recent findings on PARP1 and puts them in the context of metabolic stress and inflammation in aging.

INTRODUCTION

Aging is a multi-factorial process defined as time-dependent general decline in physiological function, which is associated with a progressively increasing risk of frailty, morbidity and mortality [1, 2]. The effect of aging is mainly observed in modern human societies and in animals under laboratory conditions [3]. The dramatic increase in mean human life span and life expectancy, coupled to a significant reduction in early mortality caused by the reduced occurrence of infections during the past two centuries, has led to an enormous increase in the number of elderly people in modern societies [4, 5]. This demographic phenomenon has been paralleled by an epidemic of chronic diseases associated with advanced age, most of which have complex etiology and underlying pathogenic mechanisms [6]. Intensive efforts have been made over the last decades to identify single key players involved in age-related diseases. Poly(ADP-ribose) polymerase 1 (PARP1) is a chromatin-associated nuclear protein which functions as stress sensor and as such is involved in the cellular responses to a variety of age-related stress signals.

Poly(ADP-ribose) polymerase 1 as molecular stress sensor

PARP1 is an abundant nuclear chromatin-associated multifunctional enzyme found in most eukaryotes apart from yeast [7]. PARP1 has been initially thought to be the only existing enzyme with poly(ADP-ribosyl)ation activity in mammalian cells. However, five additional *Parp*-like genes encoding "*bona fide*" PARP enzymes have been identified in recent years, indicating that PARP1 belongs to a family of "*bona fide*" PARP

enzymes [8]. The basal enzymatic activity of PARP1 is very low, but is stimulated dramatically under conditions of cellular stress [9, 10]. Activation of PARP1 results in the synthesis of poly(ADP-ribose) (PAR) from nicotinamide adenine dinucleotide (NAD^+) and in the release of nicotinamide as reaction by-product [7, 8]. Following PARP1 activation, intracellular PAR levels can rise 10–500-fold [11-13]. Despite intensive research on the cellular functions of PARP1, the molecular mechanism of PAR formation has not been comprehensively understood. Up to now, two different modes of PARP1 activation have been described, one dependent on DNA damage and one dependent on post-translational protein modifications (see below).

PAR is a heterogeneous linear or branched homopolymer of repeating ADP-ribose units linked by glycosidic ribose-ribose bonds [7, 9, 14]. Most free or protein-associated PAR molecules are rapidly degraded *in vivo* [15]. This rapid turnover strongly suggests that PAR levels are tightly regulated under physiological stress conditions and that degradation of the polymer starts immediately upon initiation of PAR synthesis. To date two enzymes, poly(ADP-ribose) glycohydrolase (PARG) and ADP-ribosyl protein lyase, have been described to be involved in PAR catabolism [16, 17]. While PARG possesses both exo- and endoglycosidic activities, the lyase has been described to cleave the bond between proteins and mono(ADP-ribose). The attachment of negatively charged PAR onto proteins is transient but can be very extensive *in vivo*, as polymer chains can reach more than 400 units on protein acceptors [7]. PAR formation has been implicated in a variety of cellular processes, such as maintenance of genomic stability, transcriptional regulation, energy metabolism and cell death [7]. The physiological consequences of this post-translational modification on the molecular level, however, are not yet completely understood. It has been proposed that PAR may have a dual role in modulating cell survival and cell death [9, 18, 19]. Low to moderate levels of PAR may be beneficial for important cellular functions, whereas extensive PAR formation can be detrimental and lead to various forms of cell death. More than a decade ago, PARP1 activity was linked to the aging process, as poly(ADP-ribosyl)ation capacity was shown to correlate with species-specific longevity [20, 21].

Most proteins associated with PAR are nuclear DNA-binding proteins, including PARP family members and histones [7, 22, 23]. PARP1 is the main acceptor for poly(ADP-ribosyl)ation *in vivo* and auto-modification of PARP1 abolishes its affinity for NAD^+ and DNA [24, 25]. A similar effect has been postulated for histones/nucleosomes. PAR polymers could function to alter chromatin conformation through covalent or non-covalent interactions with histone tails and via displacement of histones from DNA, thus regulating the accessibility of the genetic material. It was suggested that PAR might either directly participate in chromatin remodelling processes or indirectly coordinate them through recruitment and regulation of specific chromatin remodelling proteins [7, 22]. Moreover, PAR is recognized and bound by macrodomain containing histone variants [26].

Over 20 years ago, Nathan Berger was the first to suggest that cellular stress (e.g. oxidative damage) causes over-activation of PARP1 and subsequent NAD^+ depletion [27, 28]. In an attempt to restore the NAD^+ pools, NAD^+ is resynthesized with a consumption of 2-4 molecules of ATP per molecule of NAD^+. As a consequence, cellular ATP levels become depleted, leading to subsequent energy failure, which results in cellular dysfunction and eventually in necrotic cell death [27, 28]. Pharmacological inhibition of the enzymatic activity of PARP or the complete absence of PARP1 was shown to significantly improve cellular energetic status and cell viability after exposure to necrosis-inducing agents [29-31]. The contribution of poly(ADP-ribosyl)ation reactions to necrotic cell death seems to be dependent on the cell type and the cellular metabolic status [7, 32, 33].

Interestingly, genetic studies using *Parp1* knockout mice provided preliminary evidence that energy depletion alone might not be sufficient to mediate poly(ADP-ribosyl)ation-dependent cell death [34]. A second model has been proposed to explain how PARP1 regulates cell death. This model suggests that over-activation of PARP1 induces translocation of apoptosis-inducing factor (AIF) from the mitochondria to the nucleus, causing DNA condensation and fragmentation, and subsequent cell death [35].

Together, PARP1 can be regarded as molecular stress sensor with many physiological cellular functions. Over-activation of PARP1 results in the generation of large amounts of PAR. Subsequently, cellular NAD^+ pools are depleted and AIF is released from the mitochondria to trigger cell death. Importantly, these PARP1-dependent cellular suicide mechanisms have been implicated in the pathomechanisms of neurodegenerative disorders, cardiovascular dysfunction and various other forms of inflammation [36].

Activation of PARP1 by reactive oxygen species (ROS)

A unified theory explaining the pathogenesis of diverse degenerative conditions in different organs (including

Alzheimer's, Parkinson's and other neurodegenerative disorders, rheumatoid arthritis, atherosclerosis and other cardiovascular diseases, diabetes) has been proposed to explain how the single physiological process of aging may lead to diverse pathological states [37]. This oxidative stress theory of aging (or free radical theory of aging), initially proposed by Harman in 1956, provides the most plausible and currently acceptable global mechanism to explain the aging process [38]. The theory postulates that aging is, in the absence of other risk factors (e.g. infections, smoking, hypercholesterolemia), the net consequence of free radical-induced damage and the inability to counter-balance these changes by anti-oxidative defenses. An increase in intracellular ROS levels through hydrogen peroxide treatment of cells or through the inhibition of ROS scavenging enzymes, such as superoxide dismutase (SOD1), causes premature senescence and can shorten cellular life span [39-45]. Mitochondria are the main producers of cellular ROS under normal conditions, as approximately 1-2% of the oxygen molecules consumed during respiration are converted into highly reactive superoxide anions [46]. Besides aerobic metabolism in mitochondria, β-oxidation in peroxisomes and certain enzymes can produce ROS. Intracellular ROS can damage cellular components through oxidation of macromolecules such as nucleic acids, proteins and lipids [47]. Moreover, an overproduction of ROS leads to rapid generation of peroxinitrite from nitric oxide and superoxide, causing an imbalance in nitric oxide signaling [48].

Since the oxidative stress theory was first proposed, a considerable body of evidence has been published corroborating the idea that increased production of ROS underlies cellular dysfunction in various organ systems of aged humans and laboratory animals [49]. Interestingly, the enzymatic activity of PARP1 can be strongly activated by treatment of cells with ROS such as hydrogen peroxide [8]. Earlier studies described that PARP1 binds to oxidative damage-induced strand breaks within the DNA via two zinc finger motifs and thereby becomes activated [9]. More recently, several studies suggested that PARP1 activity is also regulated in a DNA-independent manner. A proteomic investigation uncovered many ERK1/2-induced phosphorylation sites in PARP1, which are located within important functional domains, consistent with regulatory roles *in vivo* [50, 51]. Furthermore, DNA-independent PARP1 activation can be triggered by the direct interaction of PARP1 with phosphorylated ERK-2 without PARP1 being phosphorylated itself [52]. In addition, PARP1 can be activated by elevated levels of extracellular glucose, Ca^{2+} and angiotensin II, and allosteric regulation of auto-poly(ADP-ribosyl)ation by Mg^{2+}, Ca^{2+}, polyamines, ATP and the histones H1 and H3 has been reported [53]. Whether ROS-mediated activation of PARP1 is due to ROS-generated DNA damage or also based on other ROS-induced cellular (signaling) mechanisms awaits further investigations.

PARP1 is linked to energy metabolism through NAD^+

NAD^+ biosynthesis has become of considerable interest due to the important signaling functions of pyridine nucleotides. In mammals, niacin (collectively designating nicotinamide and nicotinic acid) and the essential amino acid tryptophan are precursors of NAD^+ biosynthesis [12, 54]. The formation of dinucleotides from ATP and the mononucleotide of niacin constitute the most critical step in NAD^+ generation, which is catalyzed by NMN/NaMN adenylyltransferases (NMNATs) [13, 55]. Since PARP1 uses NAD^+ as substrate to synthesize PAR, PARP1 decisively depends on the amount of NAD^+ available and may act as energy sensor in the nucleus. Both constitutive and activated levels of PAR have been suggested to be strictly dependent on the concentration of NAD^+ in cells [15, 56, 57]. Importantly, the nuclear concentration of NAD^+ can be modulated by NMNAT-1 and a recent study revealed that NMNAT-1 is able to interact with and stimulate PARP1 [58]. It is thus tempting to speculate that PARP1 activation is supported by the localized action of NMNAT-1. Depending on the level of PARP1 activity, the cellular NAD^+ concentration is concomitantly reduced. Therefore, PARP1 not only is a sensor of NAD^+, but in turn also influences cellular energy levels.

Dietary restriction, also called calorie restriction, is defined as a life-long moderate (20-40%) reduction in caloric intake and has repeatedly been shown to extend the longevity of both invertebrates and vertebrates [59, 60]. Reducing the caloric intake starting even at an old age has also been shown to increase the life span of flies and mice and is sufficient to reverse gene expression changes associated with aging [61-63]. Furthermore, dietary restriction in rodents delays the onset and reduces the severity of many age-related diseases, such as cardiovascular disease, diabetes, osteoporosis, cataracts, neurodegenerative disease and cancers [60]. Although it was initially expected that dietary restriction would reduce overall cellular energy levels by slowing down glycolysis and the tricarboxylic acid (TCA) cycle [59], this assumption has been challenged, since dietary restriction was shown to cause an increase in NAD^+/NADH ratios in yeast cultures [64]. Whether this is also the case in mammalian cells remains to be determined. Along the same lines, the impact of dietary

restriction on enzymes that depend on NAD^+ (e.g. PARP1) is currently being investigated in multiple laboratories. Whether and how PARP1 activation differs in species with different maximal life span (and possibly also with different cellular NAD^+ pools), however, remains an open question.

Crosstalk between PARP1 and other NAD^+-consuming enzymes

NAD^+ is an essential cofactor regulating numerous cellular pathways and has recently been recognized as a substrate for a growing number of NAD^+-dependent enzymes [11, 13]. NAD^+-dependent post-translational protein modifications are catalyzed by several enzyme families, including PARPs and the sirtuin family of NAD^+-dependent class III histone deacetylases (SIRTs) [8, 65, 66]. SIRTs and the yeast homolog and founding member of the sirtuins, Sir2, are induced by dietary restriction and have been implicated in senescence and aging, although the exact mechanisms are not yet known [59, 67]. Intriguingly, ADP-ribosylation by PARP1 could modulate the NAD^+-dependent deacetylation of proteins by SIRTs via the NAD^+/nicotinamide connection. The decline of NAD^+ levels and the rise of nicotinamide upon PARP1 activation have immediate effects on other NAD^+-consuming enzymes [57, 68, 69]. SIRTs require NAD^+ as substrate and are inhibited by low levels of nicotinamide [70]. Consequently, under conditions of cellular stress and PARP1 activation, the activity of SIRTs is downregulated.

PARPs and sirtuins may not only compete for the same substrate, but might also regulate each other more directly. For instance, PARP1 and SIRT1 interact at the protein level and SIRT1 might be regulated by PARP1-dependent trans-ADP-ribosylation [7]. Another link between PAR generation and acetylation/deacetylation reactions comes from the very recent identification of three lysine residues in the auto-modification domain of PARP1 as acceptor sites for auto-ADP-ribosylation [71]. The same lysines were previously identified as targets for acetylation by p300 and PCAF [72]. Remarkably, simple addition of PCAF reduced poly(ADP-ribosyl)ation of PARP1 (own unpublished observation), suggesting that the interaction domain of PARP1 with PCAF is overlapping with the ADP-ribose acceptor sites. We recently also published that acetylation of lysine residues interferes with ADP-ribosylation [73]. This finding points at an interesting crosstalk between acetylation of and ADP-ribosylation by PARP family members. It will certainly be interesting to further investigate the crosstalk between PARP1-dependent ADP-ribosylation and acetylation/deacetylation reactions. NAD^+ levels can be expected to play an important role for the interplay between these two NAD^+-dependent post-translational protein modifications. Whether the balance between and the tight regulation of poly(ADP-ribosyl)ation and NAD^+-dependent deacetylation is altered during aging remains to be investigated. Furthermore, it will be important to identify additional NAD^+-dependent enzymes involved in the aging process.

Emerging pathological evidence indicates that major chronic age-related diseases, such as atherosclerosis, arthritis, dementia, osteoporosis and cardiovascular disease, are inflammation-related [74]. A link between NAD^+ metabolism and the regulation of an inflammatory response is suggested by the finding that nicotinamide phosphoribosyltransferase (NAMPT), one of the enzymes involved in NAD^+ biosynthesis from nicotinamide, increases cellular NAD^+ levels in response to stress [75]. The expression of NAMPT is upregulated in activated lymphocytes [76]. Furthermore, NAMPT protein and/or mRNA levels were also found to be upregulated upon stimulation of immune cells both *in vivo* and *in vitro* [77, 78], whereas a specific NAMPT inhibitor was found to inhibit cytokine production [79]. Notably, nicotinamide is known to inhibit the production of key inflammatory mediators [80-82], protects neurons against excitotoxicity [83, 84], and blocks replicative senescence of primary cells [85]. Moreover, a recent study suggested that intracellular NAD^+ levels regulate TNF-α protein synthesis in a SIRT6-dependent manner [86]. Both, SIRT1 and SIRT6 also regulate NF-κB signaling with effects on senescence and possibly aging [87, 88].

Together, accumulating evidence suggests that cellular NAD^+ biosynthesis and the NAD^+-consuming reactions poly(ADP-ribosyl)ation and SIRT-dependent deacetylation are tightly interrelated and have functions in inflammation and age-related diseases.

PARP1 is linked to age-related inflammation as transcriptional cofactor of NF-κB

A body of experimental and clinical evidence suggests that the immune system is implicated in almost all age-related or associated diseases [89, 90]. There is a well-established connection between oxidative stress and the inflammatory immune response [37]. A prominent mechanism by which age-induced ROS modulate inflammation is by inducing the redox-sensitive transcription factor nuclear factor kappa B (NF-κB). This induction of NF-κB leads to the generation of pro-inflammatory mediators and a state of chronic

inflammation [91, 92]. NF-κB plays an important role in inflammatory phenotypic changes in various pathophysiological conditions [49]. In fact, NF-κB has a fundamental role in mediating all the classical attributes of inflammation – rubor, calor, dolor and tumor – by regulating transcriptional programs in tissues containing epithelial and stromal cells, vascular endothelial cells and hematopoietic cells [93]. During the last decade, it has been clearly demonstrated that excessive activation or inappropriate regulation of immune and inflammation cascades causes tissue and cellular damage, which can lead to cellular dysfunction and death [14]. Furthermore, it was suggested that chronic, low-grade inflammation is a possible converging process linking normal aging and the pathogenesis of age-related diseases [94]. This hypothesis is in accordance with the finding that constitutive activation of NF-κB, accompanied by elevated levels of inflammatory markers, is a ubiquitous phenomenon observed in various cell types in the aging phenotype [95].

In most unstimulated cells, NF-κB is sequestered in the cytoplasm as an inactive transcription factor complex by its physical association with one of several inhibitors of NF-κB (IκB) [96-100]. The key regulatory event in NF-κB induction is the phosphorylation of IκB proteins by the IκB kinase (IKK) complex, which leads to IκB protein ubiquitylation and subsequent degradation [101, 102]. ROS have been reported to induce the activation of NIK/IKK and MAPK pathways that lead to the degradation of IκB and subsequent NF-κB-dependent gene expression [74, 103]. Conversely, induction of NF-κB itself results in the generation of ROS via the expression of inducible nitric oxide synthase (iNOS), thus activating a feedback loop that amplifies the process of damage and deterioration in target cells and organs [37].

Global screens for age-specific gene regulation have been performed from many tissues in mice and humans [3]. These analyses have recently provided evidence that the NF-κB binding domain is the genetic regulatory motif most strongly associated with the aging process and that NF-κB target genes show a strong increase in expression with age in human and mouse tissues as well as in stem cells [104-106]. Furthermore, NF-κB is implicated in age-dependent induction of cellular senescence in epithelial and hematopoietic progenitor cells [104, 107]. Blockade of NF-κB in the skin of aged mice can reverse the global gene expression program and tissue characteristics to that of younger animals [108]. Moreover, Donato et al. reported lately that in vascular endothelial cells of aged human donors nuclear NF-κB levels increase, IκBα levels decrease and that the expression of proinflammatory cytokines, such as interleukin 6 (IL-6), tumor necrosis factor-α (TNF-α) and monocyte chemoattractant protein 1 (MCP-1) is reduced [109]. NF-κB activity was also increased in aged rat vessels and kidneys, but reduced in rats under calorie restriction [110, 111].

Studies performed with *Parp1* knockout mice have identified various detrimental functions of PARP1 in inflammatory and neurodegenerative disorders. *Parp1* gene-disruption protected from tissue injury in various oxidative stress-related disease models ranging from stroke, (MPTP)-induced parkinsonism, myocardial infarction, streptozotocin-induced diabetes, lipopolysaccharide-induced septic shock, arthritis, to colitis and zymosan-induced multiple organ failure [7, 73, 112, 113]. There are striking similarities between the expression pattern of PARP1 and the detrimental transcriptional activity of NF-κB. In most tissues and cell types associated with high PARP1 expression, dysregulated NF-κB activity seems to contribute to cellular dysfunction and necrotic cell death during inflammatory disorders [14]. The strongest indication for a direct role of PARP1 in NF-κB-dependent transcription was the impaired expression of NF-κB-dependent pro-inflammatory mediators in *Parp1* knockout mice [113]. Moreover, the upregulation of several inflammatory response genes after treatment with inflammatory stimuli was drastically reduced in *Parp1* knockout mice [112, 114-116]. Our group provided first evidence that PARP1 is required for specific NF-κB-dependent gene activation and can act as transcriptional coactivator of NF-κB *in vivo* [117]. PARP1 is required and sufficient for specific transcriptional activation of NF-κB in response to pro-inflammatory stimuli and cellular stress. Furthermore, Tulin and Spradling found that Drosophila mutants lacking normal PARP levels display immune defects similar to mice lacking the NF-κB subunit p50 [118]. These results imply that the role of PARP1 in NF-κB-dependent gene expression during immune responses has been conserved during evolution. Together, several lines of evidence suggest a model in which PARP1 functions as a promoter-specific cofactor for NF-κB-dependent gene expression [7, 14].

PARP as therapeutic target for age-associated diseases

During the last two decades of intensive research, over 50 potential PARP inhibitors were developed [119]. The involvement of PARP1 in cell death (both apoptosis and necrosis) and the capacity of PARP1 to promote the transcription of pro-inflammatory genes are particularly

important for drug development. On the basis of structural information available for the catalytic domains of PARP1 and PARP2 co-crystallized with NAD^+ or certain PARP inhibitors, it became clear that the majority of PARP inhibitors mimic the nicotinamide moiety of NAD^+ and bind to the donor site within the catalytic domain [120-122]. Although the physiological functions of PARPs and poly(ADP-ribosyl)ation is still under debate, numerous experimental studies during the last years have clearly demonstrated the beneficial effects of PARP inhibition from cell culture systems to pre-clinical animal models of acute and chronic inflammation [36, 119]. For instance, Vaziri and colleagues observed an extension of cellular life span when PARP activity was inhibited [123]. In animal studies, PARP inhibition and/or PARP1 deficiency is effective in different age-related diseases [119]. The PARP inhibitor 5-AIQ has been demonstrated to attenuate the expression of P-selectin and intracellular adhesion molecule-1 (ICAM-1) as well as the recruitment of neutrophils and leukocytes into the injured lung [124, 125]. Thus, application of inhibitors reduces the degree of acute inflammation and tissue damage associated with experimental lung injury. As ROS released from the recruited leukocytes cause an upregulation of adhesion molecules, treatment with PARP inhibitors contributes to the termination of this vicious cycle and inhibits the inflammatory process. Similar to the effects of pharmacological inhibitors, *Parp1* knockout mice were found to be resistant against zymosan-induced inflammation and multiple organ failure when compared with the response of wild-type animals [126].

In murine models of arthritis, inhibition of PARP with nicotinamide delayed the onset of the disease and reduced the progress of established collagen-induced arthritis [127]. 5-iodo-6-amino-1,2-benzopyrone and PJ34, two novel PARP inhibitors, were beneficial in a mouse model of collagen-induced arthritis by reducing both the incidence of arthritis and the severity of the disease [128, 129]. Similarly, GPI 6150 was found to be highly effective in a rodent model of adjuvant-induced arthritis [130].

PARP activation also has a pathogenic role in hypertension, atherosclerosis and diabetic cardiovascular complications [119, 131]. In these diseases, the function of the vascular endothelium is impaired, resulting in a reduced ability of the endothelial cells to produce nitric oxide and other cytoprotective mediators. This then sets the stage for many manifestations of cardiovascular disease. The oxidant-mediated endothelial cell injury is dependent on PARP1 and can be attenuated by pharmacological inhibitors or genetic PARP1 deficiency [115, 132]. Furthermore, PARP inhibition improves aging-associated cardiac and endothelial dysfunction [133].

In general, the severity of many inflammatory diseases is suppressed by PARP inhibitors and the production of multiple pro-inflammatory mediators is downregulated [48]. The inhibition of PARP also reduces the formation of nitrotyrosine in inflamed tissues, an indicator of reactive nitrogen species. This finding was, at first, unexpected because PARP activation is perceived to occur downstream of the generation of oxidants and free radicals in various diseases. The mechanism is probably related to the fact that PARP inhibition reduces the infiltration of neutrophils into inflammatory sites [126]. This in turn reduces oxygen- and nitrogen-centered free-radical production. The basis for the regulation of neutrophil infiltration by PARP might be related to the reduced expression of adhesion molecules [134, 135] and/or the preservation of endothelial integrity [115, 132]. Alternatively, the reduction of nitrotyrosine could be explained by the finding that PARP1 is required for the expression of iNOS, the main producer of nitric oxide in inflamed tissues [116]. In summary, multiple studies suggest that a tight regulation of PARP activity is required to prevent a variety of age-related pathological conditions.

Role of PARP1's enzymatic activity in NF-κB - dependent gene expression

There is no consensus in the literature as to whether the modulation of NF-κB-mediated transcription by PARP1 is dependent on poly(ADP-ribosyl)ation or, alternatively, merely on the physical presence of PARP1 [14]. Genetic approaches provide strong evidence that poly(ADP-ribosyl)ation is not affecting the DNA binding activity of NF-κB and is not required for NF-κB-dependent gene expression [14, 136]. Neither the enzymatic activity of PARP1 nor its binding to DNA was required for full activation of NF-κB in response to various stimuli *in vivo* when tested on transiently transfected reporter plasmids [137, 138]. Consistently, the enzymatic activity of PARP1 was not required for full transcriptional activation of NF-κB in the presence of the histone acetyltransferase p300 [72]. At first glance this seems not to be compatible with reports describing that PARP inhibitors abolish mRNA expression of iNOS, IL-6 and TNF-α in cultured cells [139] or that PARP inhibitors reduce the expression of inflammatory mediators in mice [124, 126, 140]. However, this discrepancy might be explained in three ways: First, it should be noted that the currently available PARP inhibitors do not discriminate well between PARP1 and other PARP family members or

even other NAD^+-metabolizing enzymes, which are described to also play a role in inflammatory response pathways [139, 141]. In *Parp1* knockout mice, PAR formation is indeed drastically reduced only in brain, pancreas, liver, small intestine, colon, and testis, whereas still moderate levels of residual poly(ADP-ribose) formation can be observed in the stomach, bladder, thymus, heart, lung, kidney and spleen [7]. This residual activity can most likely be attributed to PARP2, which has the greatest similarity to PARP1 among all PARP family members [8]. Interestingly, PARP2 is involved in T lymphocyte development and survival [142] and has been implicated in inflammatory immune responses [143, 144]. A putative role of PARP2 in aging awaits further investigations. Second, based on recent reports, one cannot exclude the possibility that PARP-inhibitors might even affect non-NAD^+-consuming targets such as AKT/PKB or MMPs [145]. Third, the enzymatic activity of PARP1 might be required for the transcriptional activity of transcription factors involved in inflammatory processes other than NF-κB. Several groups have shown that co-operative activities between transcription factors such as AP-1, STAT-1 or IRF-1 in the enhanceosomes of NF-κB dependent genes are required for full synergistic activation of target genes [146, 147]. Considering these constraints of all currently available PARP inhibitors, the specific contribution of PARP1 enzymatic activity for age-related diseases, in which PARP inhibition has beneficial effects, needs to be evaluated very carefully.

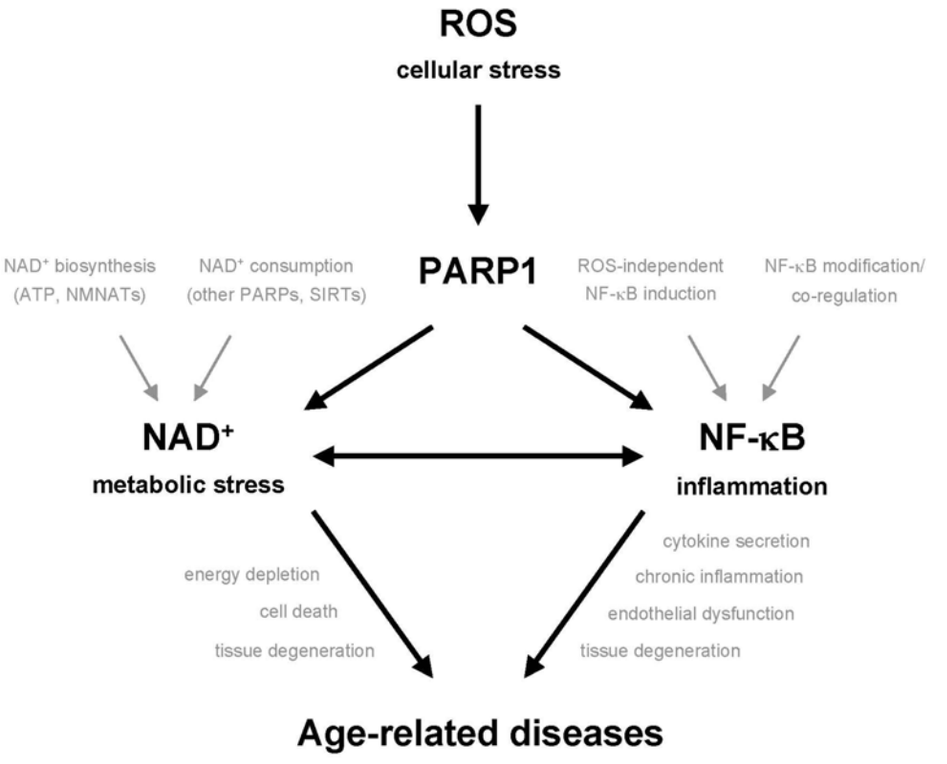

Figure 1. PARP1 at the crossroad of metabolic stress and inflammation in aging. PARP1 is activated by cellular stress, e.g. by oxidative damage due to increased levels of reactive oxygen species (ROS). As NAD^+-dependent enzyme, PARP1 senses energy levels and crosstalks with other NAD^+-consuming enzymes. Over-activation of PARP1 leads to energy depletion and cell death. On the other hand, PARP1 functions as cofactor for NF-κB-dependent transcription and is therefore implicated in many inflammatory processes. Both, PARP1-mediated metabolic stress and PARP1-regulated inflammation can lead to tissue degeneration underlying many age-related pathologies. See text for further details.

CONCLUSIONS

Several publications in the past years indicate that the nuclear protein PARP1 represents a molecular link between energy metabolism and inflammation (Figure). As NAD^+-consuming enzyme, PARP1 acts as nutrient or energy sensor, crosstalks with other NAD^+-consuming enzymes (such as sirtuins) and modulates (as regulator of NF-κB-dependent transcription of cytokines) inflammatory responses. Thus, PARP1 seems to be an ideal candidate to integrate metabolic and inflammatory signals, which arise during the process of aging. As central integrator, PARP1 may mediate cellular stress response pathways and thereby participate in a multitude of age-related pathologies. PARP inhibition has proven beneficial in many cell culture and animal model systems of acute and chronic inflammation and age-related diseases. Clearly, additional research will further improve our understanding of the functions of PARP1 and their implications in age-related diseases associated with metabolic stress and inflammation.

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Molecular mechanism of poly(ADP-ribosyl)ation by PARP1 and identification of lysine residues as ADP-ribose acceptor sites

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ABSTRACT

Poly(ADP-ribose) polymerase 1 (PARP1) synthesizes poly(ADP-ribose) (PAR) using nicotinamide adenine dinucleotide (NAD) as a substrate. Despite intensive research on the cellular functions of PARP1, the molecular mechanism of PAR formation has not been comprehensively understood. In this study, we elucidate the molecular mechanisms of poly(ADP-ribosyl)ation and identify PAR acceptor sites. Generation of different chimera proteins revealed that the amino-terminal domains of PARP1, PARP2 and PARP3 cooperate tightly with their corresponding catalytic domains. The DNA-dependent interaction between the amino-terminal DNA-binding domain and the catalytic domain of PARP1 increased V_{max} and decreased the K_m for NAD. Furthermore, we show that glutamic acid residues in the auto-modification domain of PARP1 are not required for PAR formation. Instead, we identify individual lysine residues as acceptor sites for ADP-ribosylation. Together, our findings provide novel mechanistic insights into PAR synthesis with significant relevance for the different biological functions of PARP family members.

INTRODUCTION

Poly(ADP-ribose) polymerases (PARPs) use nicotinamide adenine dinucleotide (NAD) as substrate to synthesize poly(ADP-ribose) (PAR) (1). On the cellular level, PAR formation has been implicated in a wide range of processes, such as maintenance of genomic stability, transcriptional regulation, energy metabolism and cell death (2).

PARP1 was the first protein described to catalyze PAR formation in response to mitogenic stimuli or genotoxic stress (3–7). It contains three functionally distinct domains: an amino-terminal DNA-binding domain (DBD), an auto-modification domain (AD) and a carboxyl-terminal PARP homology domain that includes the catalytic domain (CAT) responsible for PAR formation (8). The DBD extends from the initiator methionine to threonine 373 in human PARP1. It contains two structurally and functionally unique zinc fingers (FI: aa, amino acid, 11–89; FII: aa 115–199) (2,9). Recently, a third and so far unrecognized zinc-binding motif was discovered (FIII: aa 233–373) (10,11). The DBD also contains a bipartite nuclear localization signal (NLS) of the form KRK-X(11)-KKKSKK (aa 207–226) that targets PARP1 to the nucleus (12). The PARP1 zinc fingers FI and FII are thought to recognize altered structures in DNA rather than particular sequences and have also been reported to be involved in protein–protein interactions (13). PARP1 strongly associates with DNA single and double strand breaks generated either directly by DNA damage or indirectly by the enzymatic excision of damaged bases during DNA repair processes (2,9). Several studies indicate that the first zinc finger is required for PARP1 activation by both DNA single and double strand breaks, whereas the second zinc finger may exclusively act as a DNA single strand break sensor (2,9).

The AD of PARP1 is located in the central region of the enzyme, between residues 373 and 525 of human PARP1 (14,15). It was identified as the domain containing acceptor amino acids for the covalent attachment of PAR (16). In addition, several recent studies identified a weak leucine-zipper motif in the amino-terminal region of the AD, which suggests that this motif might be involved in homo- and/or hetero-dimerization (9). The AD of PARP1 also comprises a breast cancer 1 protein (BRCA1) C-terminus (BRCT) domain (from aa 386 to 464 in

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human PARP1) as well as an unstructured loop that connects the AD with the PARP homology domain.

PARP1 contains an 80–90 amino acid long tryptophane-, glycine-, arginine-rich (WGR) domain carboxyl terminal of the AD. The WGR domain is named after the most conserved central motif of tryptophane (W), glycine (G), arginine (R) residues and may represent a nucleic-acid-binding domain (2). This region of PARP1 has not been extensively characterized and its function is still unknown. The CAT has been suggested to catalyze at least three different enzymatic reactions: the attachment of the first ADP-ribose moiety onto an acceptor amino acid (initiation reaction), the addition of further ADP-ribose units onto already existing ones (elongation reaction) and the generation of branching points (branching reaction) (8). The active site is formed by a phylogenetically well-conserved sequence of ~50 residues (aa 859–908 of hPARP1). This 'PARP signature' contains the NAD acceptor sites and critical residues involved in the initiation, elongation and branching of PAR.

Like PARP1, both PARP2 and PARP3 also contain a WGR as well as a CAT (16). PARP2 and PARP3 lack, however, most motifs present in the amino-terminal half of PARP1. Neither zinc-binding motifs nor leucine-zippers or BRCT domains have been described for PARP2 or PARP3. PARP2 contains an amino-terminal SAP/SAF motif/module [named after scaffold-associated protein/scaffold-associated factor SAF-A/B, Acinus and PIAS; (17)] and a eukaryotic module proposed to be involved in sequence- or structure-specific DNA and RNA binding (18). Furthermore, PARP2 contains an amino-terminal NLS which targets the protein to the nucleus. PARP3 is the least studied and smallest PARP identified so far (19). The protein domain structure of PARP3 is very similar to that of PARP2, featuring a small putative DBD consisting of only 54 residues and apparently containing a targeting motif that is sufficient to localize the enzyme to the centrosome (19,20).

Attempts to obtain structural information on the full-length proteins PARP1, PARP2 and PARP3 by X-ray crystallography or by nuclear magnetic resonance (NMR) have not been successful up to now. The 3D structures of single domains, however, have been solved and allow for a structure-based comparison of different PARP family members (8,21) (PDB: 1A26, 1GS0 and 2PA9). Although the amino acid identity between PARP1 and PARP2 or PARP3 is only moderate (40% and 32% in the CAT, respectively), the overall structure of the CATs of these three proteins is nearly identical. This conservation suggests, in general, similar capabilities to generate PAR. Both PARP1 and PARP2 have been shown to synthesize very complex branched polymers at least *in vitro* (2). The enzymatic activity of PARP3 and its isoforms has not yet been investigated in detail.

An unresolved issue regarding the mechanism of poly(ADP-ribosyl)ation is how DNA binding in the amino-terminal DBD triggers enzyme activation in the carboxyl-terminal CAT and how the different domains of the different PARPs are coordinated during this process. Furthermore, earlier studies suggested that the automodification activity targets between 4 and 28 acceptor residues located in the AD and in the DBD of PARP1 (14,22,23). For histone H1, a major target for trans-poly(ADP-ribosyl)ation by PARP1, glutamic acid residues have been described to function as acceptors for PAR (24). This, together with the reported chemical similarity between the ADP-ribose-PARP1 linkage and carboxyl esters in mono-ADP-riboslyated histones (23), led to the hypothesis that multiple glutamic acid residues present in the AD of PARP1 might function as acceptor sites for auto-poly(ADP-ribosyl)ation (16). However, despite intensive research during the last 40 years, the acceptor amino acids in PARP1 have not been confirmed by mutational studies.

Here, we comprehensively analyze PAR formation by PARP1, PARP2 and PARP3 and find a close cooperativity between the amino-terminal portions of the proteins and their corresponding CATs. We define the DBD (aa 1–373) and the WGR/CAT domain (aa 533–1014) as the minimal domains of PARP1 required for PAR formation. The DNA-dependent interaction between the DBD and the CAT increased V_{max} and decreased the K_m for NAD. Furthermore, by amino-acid substitutions, we establish that glutamic acid residues within the AD are not required for PAR formation and thus do not function as acceptor amino acids for PAR. Instead, we identify lysine residues within the AD of PARP1 as acceptor sites for ADP-ribosylation.

MATERIALS AND METHODS

Chemicals and antibodies

^{3}H-NAD and protein A sepharose were purchased from GE Healthcare and ^{32}P-NAD was from PerkinElmer. NAD was obtained from Sigma-Aldrich. Anti-PAR antibody LP96-10 was from Alexis Biochemicals or Becton Dickinson, anti-PARP1$_{cat}$ antibody H250 from Santa Cruz Biotechnology and anti-haemagglutinin (HA) antibody 16B12 from Covance.

Plasmids

The baculovirus expression vectors pQE-TriSystem (Qiagen) and BacPak8 (Clontech) were used for the expression of recombinant proteins in *Sf21* insect cells as described previously (25,26).

Cloning, expression and purification of recombinant proteins

Wild-type hPARP1 (NCBI ID: BC037545), hPARP2 (NCBI ID: NM_001042618) and hPARP3 (NCBI ID: BC014260) were cloned and expressed as carboxyl-terminal His-tagged proteins. PARP family chimera were generated by overlapping polymerase chain reaction (PCR) at the position corresponding to amino acid 533 in hPARP1 and expressed as carboxyl-terminal His-tagged proteins. Protein fragments and deletion mutants were generated by PCR and expressed as carboxyl-terminal His-tagged proteins as described before (25,26). Amino-acid substitutions were introduced by site-directed PCR-based mutagenesis and mutant proteins

were expressed as described before (25,26). All recombinant proteins were purified by one step affinity chromatography using ProBond resin according to the manufacturer's recommendations (Invitrogen). Expression and purification of all recombinant proteins was analyzed by SDS–PAGE followed by coomassie staining. For the stacking gel a 4.5% acrylamide-bis solution [37.5:1, 40% (w/v), Serva] and for the separating gel a 10–12.5% acrylamide-bis solution was used.

PAR formation assays

^{3}H-NAD time course experiments. One hundred picomoles recombinant purified enzyme and 5 µg of protein fragments in PAR reaction buffer (50 mM Tris–HCl pH 8.0, 4 mM $MgCl_2$, 250 µM DTT, 1 µg/ml pepstatin, 1 µg/ml bestatin, 1 µg/ml leupeptin) in the presence of 50 pmol annealed double-stranded oligomer (5′-GGAAT TCC-3′) were supplemented with ^{3}H-NAD to a final concentration of 400 µM. PAR formation was allowed for 1, 3, 5, 15 and 60 min at 30°C. Reactions were stopped by addition of ice-cold 10% TCA/2% $Na_4P_2O_7$. Polymers were precipitated for 10 min on ice and then applied onto filter papers. Counts per minute were obtained by liquid scintillation counting. For the determination of V_{max} and K_m, initial reaction velocities (V_0) were obtained by measuring PAR levels generated after 0, 1, 3 and 5 min incubation at different ^{3}H-NAD concentrations and using the GraphPad Prism software for nonlinear regression analysis assuming a one-site binding model. V_{max} and K_m were calculated from V_0 according to Michaelis–Menten.

Anti-PAR western blot. Unless otherwise stated, 10 pmol recombinant purified enzyme and 0.5 µg of protein fragments in PAR reaction buffer in the presence of 5 pmol annealed double-stranded oligomer (5′-GGAATTCC-3′) were supplemented with NAD to a final concentration of 400 µM. PAR formation was allowed for 5 min at 30°C. Reactions were stopped by addition of SDS–PAGE loading buffer and boiling for 5 min at 95°C. Samples were subjected to SDS–PAGE followed by anti-PAR western blot.

^{32}P-NAD auto-modification. Unless otherwise stated, 10 pmol recombinant purified enzyme and 0.5 µg of protein fragments in PAR reaction buffer in the presence of 5 pmol annealed double-stranded oligomer (5′-GGAATT CC-3′) were supplemented with ^{32}P-NAD to a final concentration of 100 nM. Auto-modification was allowed for 10 s at 30°C. Reactions were stopped by addition of SDS–PAGE loading buffer and boiling for 5 min at 95°C. Samples were subjected to SDS–PAGE followed by detection of auto-modification by autoradiography.

PAR detection by silver staining. Following synthesis of PAR as for western blot analysis, PAR chains were purified and separated by modified DNA sequencing gel electrophoresis as described by Fahrer *et al.* (27).

In vitro *co-immunoprecipitation*. Ten picomoles recombinant purified enzyme and 0.5 µg of protein fragments were incubated for 5 min at 30°C in Co-IP buffer (50 mM Tris–HCl pH 8.0, 100 mM NaCl, 4 mM $MgCl_2$, 0.2% NP-40, 250 µM DTT, 1 µg/ml pepstatin, 1 µg/ml bestatin, 1 µg/ml leupeptin) in the absence or presence of 5 pmol annealed double-stranded oligomer (5′-GGAATTCC-3′). The CAT of PARP1 was allowed to bind to the anti-PARP1$_{cat}$ antibody for 1 h at 4°C. Protein A sepharose was added and samples were incubated for another 2 h at 4°C. Samples were washed three times for 5 min in Co-IP buffer containing 300 mM NaCl before being subjected to SDS–PAGE followed by western blot.

RESULTS

Purified full-length human PARP1 and PARP2 are enzymatically active

In order to gain detailed insights into the mechanism of PAR formation by different PARP family members, we expressed and purified full-length human PARP1, PARP2 and PARP3 using the baculovirus expression system (Figure 1A and B). PARP3 showed a slower migration velocity than predicted in SDS–PAGE, possibly due to the high content of hydrophobic amino acids in the CAT. To measure PAR formation, the purified proteins were incubated for different time periods with 400 µM tritium-labeled NAD in the presence of double strand break mimicking DNA. Reaction products were precipitated by trichloroacetic acid (TCA) before they were analyzed using a beta counter. PARP1 generated PAR in a time-dependent manner (Figure 1C, left panel). PARP2 also synthesized PAR in a time-dependent manner, however, not as efficiently as PARP1 (Figure 1C, middle panel). The reduced amount of product formed by PARP2 most probably represents a quantitative rather than a qualitative difference, since the length distribution of PAR chains formed by PARP2 was comparable to the length distribution of PAR formed by PARP1 (Supplementary Figure 4B). Human PARP3 did not produce detectable amounts of PAR under the tested conditions (Figure 1C, right panel).

Assuming that mono(ADP-ribosyl)ation preceeds PAR formation, we assessed the auto-modification of the three proteins after 10 s incubation with 100 nM radiolabeled NAD (Figure 1D). The short incubation period and the low concentration of NAD were chosen to prevent polymer formation. The discrete bands observed using this approach indeed suggest that under these conditions mostly mono(ADP-ribosyl)ation occurred. In line with the time-course experiments, PARP1 and PARP2 were able to auto-modify themselves in an NAD- and DNA-dependent manner while PARP3 was not (Figure 1D).

PARP1 synthesized increasing PAR levels in a time- and DNA-dependent manner detected also by western blot and vacuum slot blot using 400 µM NAD (Supplementary Figure 1A and B). PAR formation after 5 min incubation with 400 µM NAD caused a pronounced shift of the coomassie blue-stained proteins in the denaturing gel due to a severely reduced migration velocity of the poly(ADP-ribosyl)ated proteins when

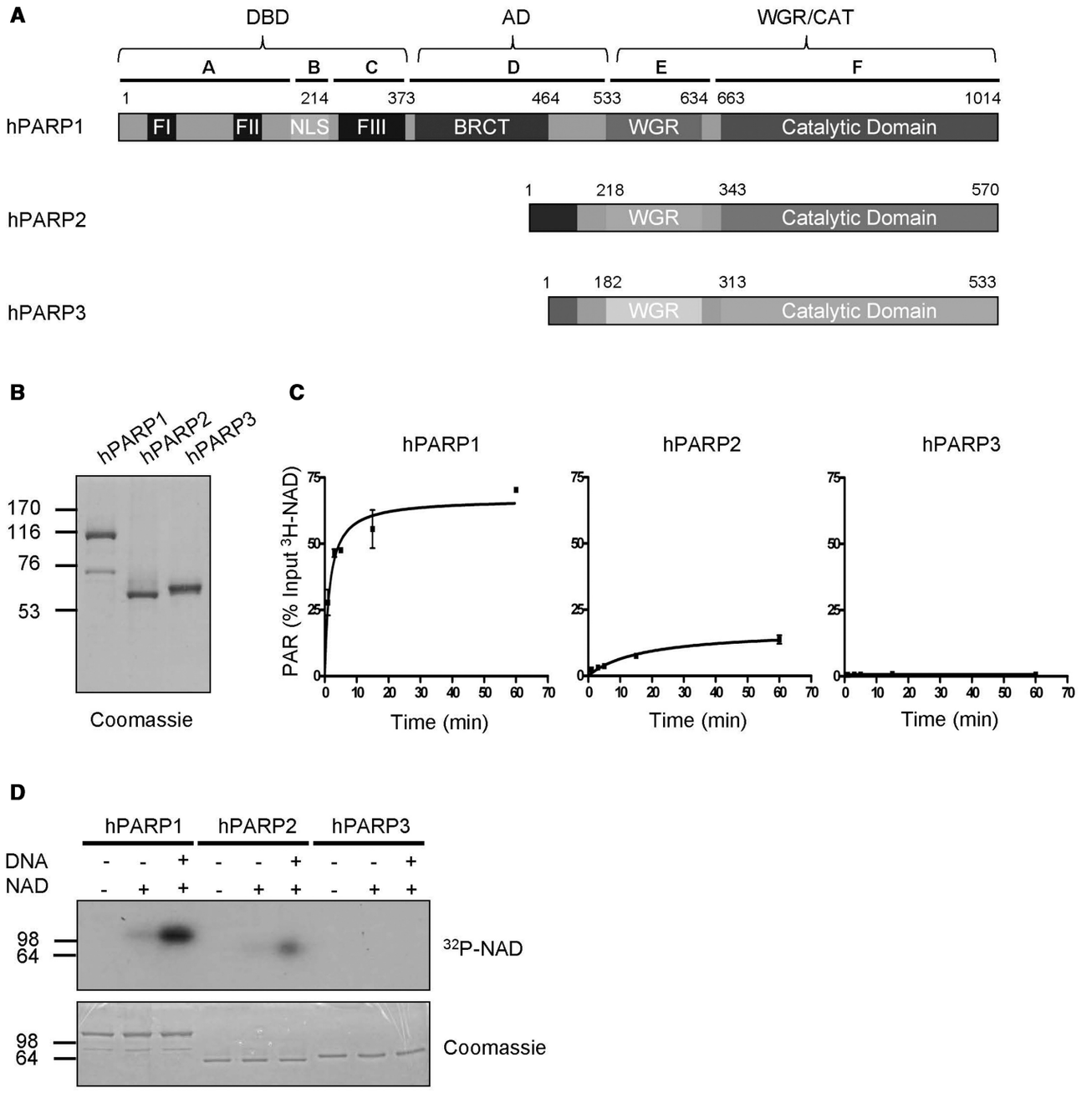

Figure 1. Purified full-length human PARP1 and PARP2 are enzymatically active. (**A**) Domain organization of human PARP1, PARP2 and PARP3. Letters A–F indicate domain nomenclature of PARP1 and numbers indicate amino acid positions. (**B**) Purity of PARP family members after one step affinity chromatography. One microgram of each recombinant, purified protein was used for SDS–PAGE followed by coomassie staining. (**C**) Time course of PAR formation by different PARP family members. ^{3}H-NAD incorporation into TCA-precipitable polymers was determined by scintillation counts. Substrate concentration: 400 μM ^{3}H-NAD. Reactions were performed in triplicates, error bars represent standard deviations. (**D**) Automodification of different PARP family members detected by autoradiography. Substrate concentration: 100 nM ^{32}P-NAD. Molecular size markers in kilo Daltons are indicated.

compared to unmodified proteins (Supplementary Figure 1C). The observed basal activity of PARP1 in the absence of DNA can be explained either by a contamination with DNA or by the intrinsic DNA-independent activity of the CAT as described by Simonin *et al.* (28). Analysis of PAR formation by silver staining after modified DNA sequencing gel electrophoresis confirmed PAR formation by PARP1 and PARP2 and no PAR formation by PARP3 (Supplementary Figure 4B).

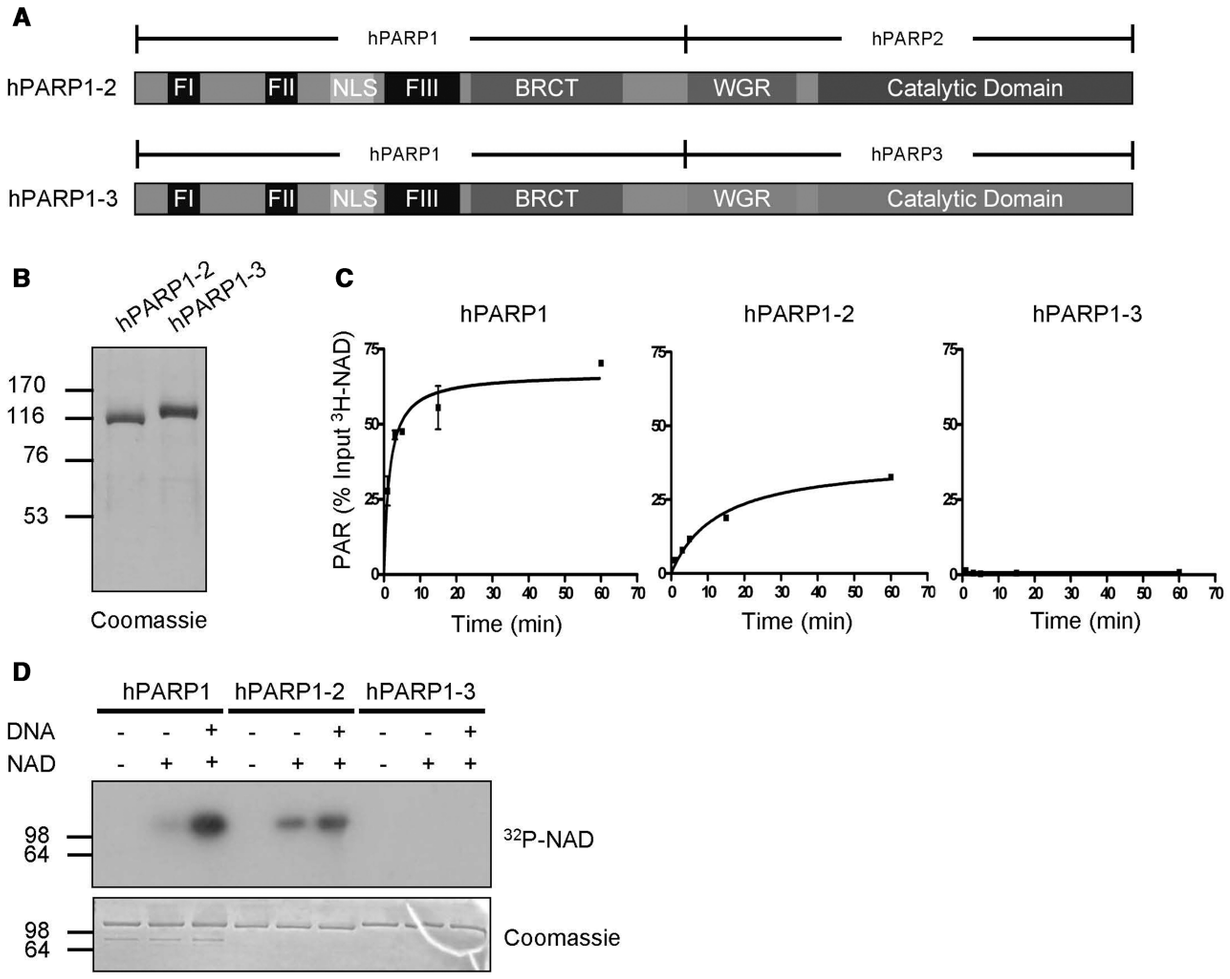

Figure 2. The carboxyl-terminal domains of PARP1, PARP2 and PARP3 cannot compensate for each other. (**A**) Domain organization of chimera PARP1-2 and chimera PARP1-3. (**B**) Purity of chimera PARP1-2 and chimera PARP1-3 after one-step affinity chromatography. One microgram of each recombinant, purified protein was used for SDS–PAGE followed by coomassie staining. (**C**) Time course of PAR formation by PARP1, chimera PARP1-2 and chimera PARP1-3 as in Figure 1C. (**D**) Auto-modification of PARP1, chimera PARP1-2 and chimera PARP1-3 detected by auto-radiography as in Figure 1D. Molecular size markers in kilo Daltons are indicated.

The carboxyl-terminal domains of PARP1, PARP2 and PARP3 cannot compensate for each other

Next, we investigated the crosstalk between the different amino-terminal domains of PARP1, PARP2 and PARP3 with their carboxyl-terminal domains (i.e. WGR/CAT). Therefore, we generated chimera proteins by replacing the WGR/CAT domain of PARP1 with the WGR/CAT domain of PARP2 or PARP3 [named chimera PARP1-2 (aa 1–532 of PARP1 fused to aa 81–570 of PARP2) or chimera PARP1-3 (aa 1–532 of PARP1 fused to aa 48–533 of PARP3)], respectively (Figure 2A and B). We analyzed PAR formation by these proteins and found that replacing the WGR/CAT domain of PARP1 by the one of PARP2 (i.e. chimera PARP1-2) resulted in an active enzyme that showed roughly similar PAR formation in time course experiments as PARP2 (Figure 2C, middle panel). Replacement of the WGR/CAT domain of PARP1 by that of PARP3 (i.e. chimera PARP1-3) resulted in an enzyme that did not produce detectable amounts of PAR under the tested conditions (Figure 2C, right panel and Supplementary Figure 1D). In line with these findings, chimera PARP1-2 was able to auto-modify itself whereas chimera PARP1-3 was not (Figure 2D). Together these results suggest that the WGR/CAT domains of the investigated PARP proteins cannot compensate for each other. The WGR/CAT domains cooperate tightly with their corresponding amino-terminal domains and limit poly(ADP-ribosyl)ation capacity and the ability for auto-modification, despite high levels of structural similarity between the CATs (see Supplementary Figure 2A).

The carboxyl-terminal domain of PARP1 is not activated by the amino-terminal domains of PARP2 or PARP3

In a second set of chimera proteins we fused the WGR/CAT domain of PARP1 to the amino-terminal domains of PARP2 or PARP3, or deleted the amino-terminal domain

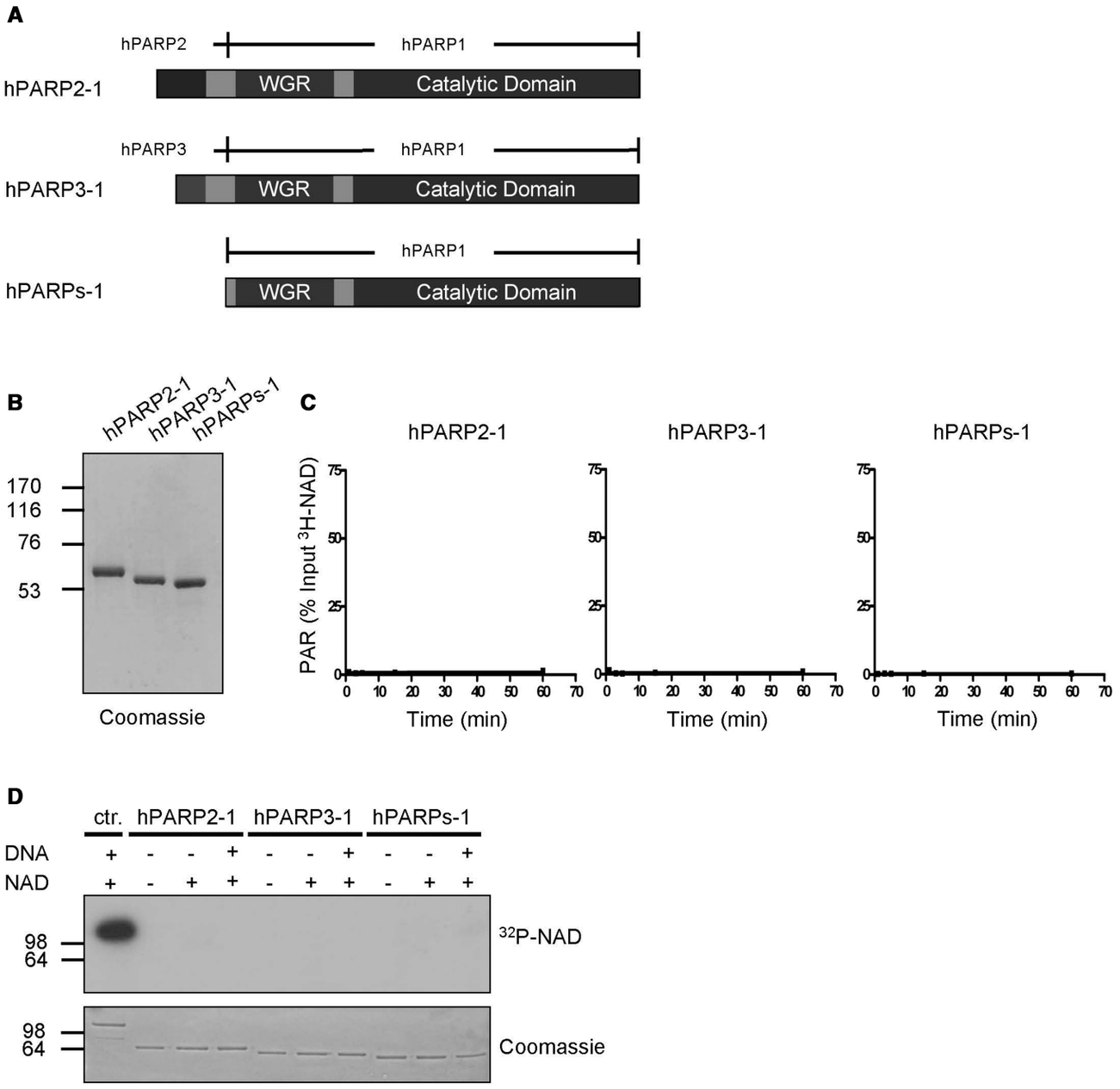

Figure 3. The carboxyl-terminal domain of PARP1 is not activated by the amino-terminal domains of PARP2 or PARP3. (**A**) Domain organization of chimera PARP2-1, chimera PARP3-1 and PARPs-1. (**B**) Purity of chimera PARP2-1, chimera PARP3-1 and PARPs-1 after one-step affinity chromatography. One microgram of each recombinant, purified protein was used for SDS–PAGE followed by coomassie staining. (**C**) Time course of PAR formation by chimera PARP2-1, chimera PARP3-1 and PARPs-1 as in Figure 1C. (**D**) Auto-modification of PARP1 (ctr.), chimera PARP2-1, chimera PARP3-1 and PARPs-1 detected by autoradiography as in Figure 1D. Molecular size markers in kilo Daltons are indicated.

of PARP1 [named chimera PARP2-1 (aa 1–80 of PARP2 fused to aa 533–1014 of PARP1), chimera PARP3-1 (aa 1–47 of PARP3 fused to aa 533–1014 of PARP1) or PARPs-1 (aa 533–1014 of PARP1)], respectively (Figure 3A and B). Analysis of these proteins revealed that chimera PARP2-1, chimera PARP3-1 and PARPs-1 did not generate detectable levels of PAR (Figure 3C and Supplementary Figure 1E). Furthermore, no auto-modification of the three proteins was observed under the tested conditions (Figure 3D). These results indicate that the WGR/CAT domain of PARP1 is only stimulated by its corresponding amino-terminal domain, but not by the amino-terminal domains of PARP2 or PARP3.

The DBD of PARP1 is sufficient to stimulate its WGR/CAT domain

To further investigate the cooperativity between the amino-terminal domain of PARP1 and its WGR/CAT

domain, the inactive chimera PARP2-1 was co-incubated with the PARP1 E988K mutant, which lacks the ability to generate PAR. Surprisingly, co-incubation of chimera PARP2-1 with PARP1 E988K strongly induced PAR synthesis, suggesting that PARP1 E988K was able to stimulate the WGR/CAT domain of chimera PARP2-1 (Figure 4A). To map the minimal domain of PARP1, which was able to stimulate the WGR/CAT domain of chimera PARP2-1, we expressed and purified different fragments of PARP1 covering all domains from A to F (see Figure 1A). Analysis of PAR synthesis by western blot upon co-incubation of chimera PARP2-1 with these fragments revealed that the DBD of PARP1 comprising amino acid 1–373 was the only fragment able to stimulate chimera PARP2-1 (Figure 4B). Further dissection of the DBD revealed that only the complete and undisrupted DBD from amino acid 1 to 373 containing FI, FII and FIII was able to stimulate chimera PARP2-1 (Figure 4C). The stimulation of chimera PARP2-1 by the DBD was salt resistant up to 300 mM NaCl (Supplementary Figure 3B, left panel).

To further assess the specificity of the observed stimulation, the DBD was incubated with different proteins (chimera PARP2-1, chimera PARP3-1, PARPs-1 and PARP1 656–1014) and the time course of PAR formation was analyzed using tritium-labeled NAD. Of note, besides chimera PARP2-1, only chimera PARP3-1 and PARPs-1, but neither the CAT of PARP1 nor full-length PARP2, PARP3, chimera PARP1-2 or chimera PARP1-3, were stimulated by the DBD of PARP1 (Figure 4D and Supplementary Figure 4A and C). Analysis of PAR formation by silver staining after polymer separation using modified DNA sequencing gel electrophoresis confirmed that the observed stimulation in the time course experiments correlated with the synthesis of PAR containing 1 to more than at least 50 ADP-ribose units (Supplementary Figure 4B).

The stimulation of chimera PARP2-1 by the DBD was strongly dependent on DNA (Figure 4E, left panel), which suggests that DNA tightly regulates the interaction necessary for the activation of the CAT. Furthermore, our observation that PARPs-1 but not PARP1 656–1014 together with the DBD was able to generate PAR indicates that the WGR domain of PARP1 is absolutely essential for enzymatic activity.

Since chimera PARP2-1 does not exist physiologically, PAR synthesis by a PARP1 DBD deletion mutant (aa 373–1014) co-incubated with the DBD was analyzed. Interestingly, the DBD was able to stimulate PARP1 373–1014 in a DNA-dependent manner and comparable to PARP2-1 (Figure 4E, right panel and Supplementary Figure 3B, right panel), suggesting that the observed stimulation of PARP2-1 by the DBD represents a physiological regulatory mechanism in the PARP1 full-length context.

The DBD of PARP1 interacts with its CAT domain

The results described above suggest that the DBD of PARP1 interacts with the CAT and/or the WGR domain to stimulate PAR synthesis by the CAT. To test this hypothesis experimentally, *in vitro* co-immunoprecipitation assays were performed with purified proteins and fragments. The complete DBD (aa 1–373), but not aa 1–214 alone, specifically bound to chimera PARP2-1 in a manner that was stabilized by DNA (Figure 4F). Similarly, the DBD also bound to PARP1 373–1014, and this interaction was enhanced by DNA (Figure 4G, left panel). Interestingly, the CAT domain of PARP1 without the WGR (aa 656–1014) was sufficient for the DNA-dependent interaction with the DBD (Figure 4G, right panel). Since PAR formation was only observed when combining the DBD with PARPs-1 (expressing WGR/CAT) but not with the CAT domain of PARP1 alone (Figure 4D and Supplementary Figure 4A), we conclude that an intact DBD (aa 1–373) interacts with the CAT in a DNA-dependent manner and that the WGR domain is additionally required to allow PAR formation.

The DBD bound to DNA activates the CAT by increasing V_{max} and decreasing K_m

Next, we determined the enzymatic parameters of chimera PARP2-1 stimulated by the PARP1 DBD in the absence or presence of DNA. We measured the incorporation of tritium-labeled NAD into TCA-precipitable polymers at early reaction time points and obtained initial reaction velocities (V_0) for different substrate concentrations by nonlinear regression analysis assuming one substrate-binding site. In the absence of the DBD, chimera PARP2-1 did not generate detectable levels of PAR independent of the addition of DNA (Table 1, second and third column), thus confirming our previous results. In the presence of the DBD, PAR generation was strongly dependent on DNA. Without DNA, the obtained PAR levels were low, but still allowed for curve fitting and calculation of V_{max} and K_m values (Table 1, fourth column). Addition of DNA increased the maximum reaction velocity V_{max} about 4-fold and reduced K_m 8-fold (Table 1, compare fifth to fourth column). The reaction efficiency K_{cat}/K_m was thereby increased by more than 30-fold. DNA could thus be considered a $V + K$-type activator, affecting both turnover rate and substrate affinity. Remarkably, the enzymatic parameters obtained for chimera PARP2-1 together with the PARP1 DBD closely match the values reported for full-length PARP1 (Table 1, compare fifth to first column). Together, these results provide evidence that DNA containing double strand breaks is recognized and bound by the DBD of PARP1, which subsequently binds to the CAT domain to induce structural changes within the catalytic cleft in order to increase the affinity for NAD and stabilize reaction intermediates.

PARP1 forms a catalytic dimer which requires at least one functional FI and FIII domain for activity

The CAT of PARP1 was previously described to dimerize (29). To investigate whether our purified proteins were also able to form dimers, the enzymatic activity of full-length PARP1 was assessed by western blot analysis after co-incubation with different molar ratios of two catalytically inactive PARP1 mutants (E988K or M890V/D899N,

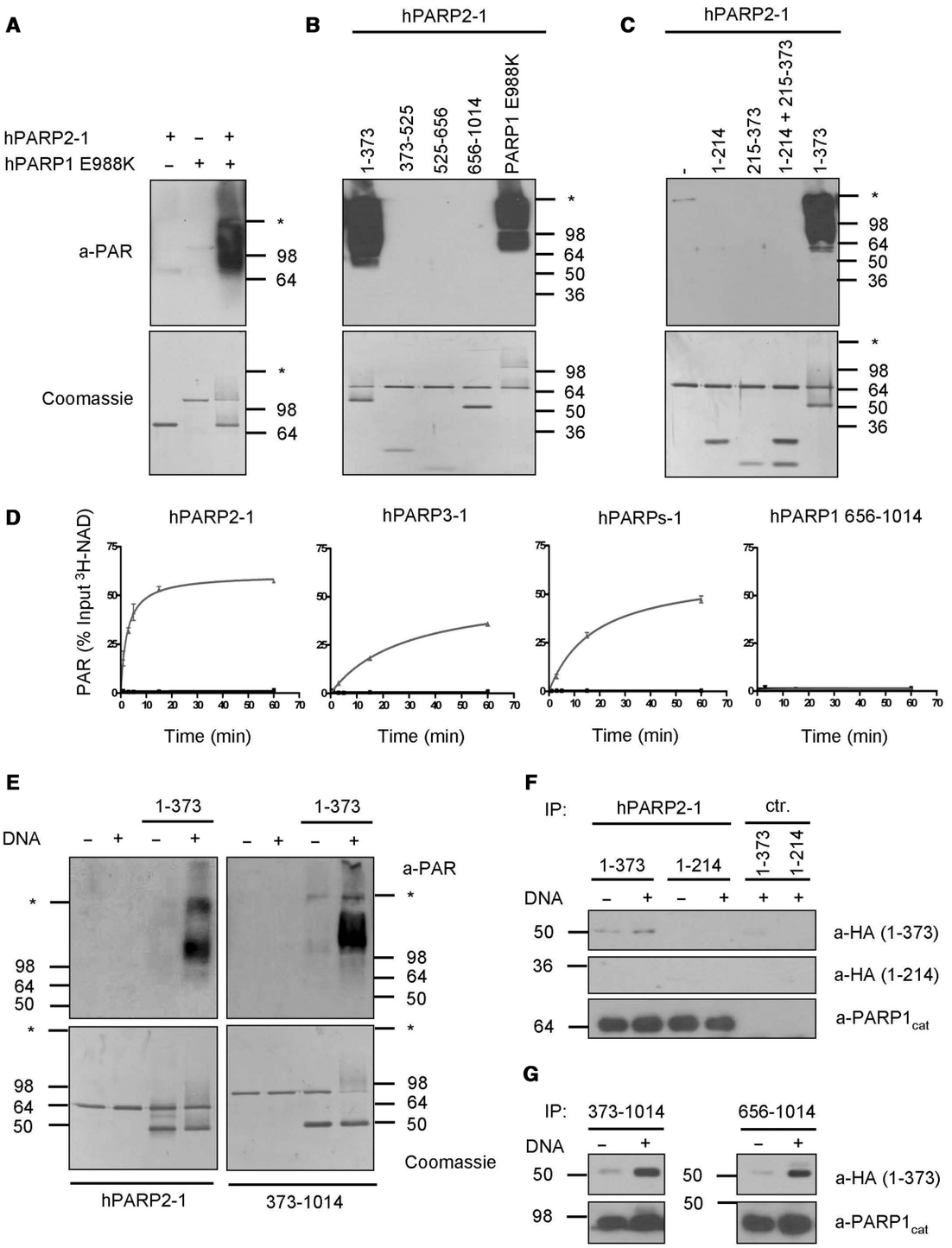

Figure 4. The DBD of PARP1 interacts with and is sufficient to stimulate its WGR/CAT domain. (**A**) PAR formation by chimera PARP2-1 co-incubated with catalytically inactive PARP1 E988K. PAR was detected by western blot using anti-PAR antibody LP96-10. Substrate concentration: 400 μM NAD. (**B**) PAR formation by chimera PARP2-1 co-incubated with the indicated fragments of PARP1 or with PARP1 E988K. (**C**) PAR formation of chimera PARP2-1 co-incubated with the indicated fragments or combination of fragments of PARP1. (**D**) Time course of PAR formation by chimera PARP2-1, chimera PARP3-1, PARPs-1 and PARP1 656–1014 in the absence or presence of fragment 1–373 as in Figure 1C. *Black* without fragment 1–373 and *grey* with fragment 1–373. (**E**) PAR formation of chimera PARP2-1 or PARP1 373–1014 co-incubated with fragment 1–373 in the absence or presence of DNA. (**F**) *In vitro* interaction between chimera PARP2-1 and 1–373. Chimera PARP2-1 was bound to protein A sepharose using an antibody against the CAT of PARP1 (a-PARP1$_{cat}$) and was then incubated with HA-tagged fragment 1–373 or 1–214 in the absence or presence of DNA. HA-tagged fragments were detected by western blot. PARP1$_{cat}$ antibody coupled to beads without chimera PARP2-1 served as control (ctr.). (**G**) *In vitro* interaction between PARP1 373–1014 or 656–1014 with 1–373. Experiments were performed as described in (F). Molecular size markers in kilo Daltons and the border between stacking and separating gel (*asterisk*) are indicated.

Table 1. Kinetic parameters of chimera PARP2-1

	hPARP1[a]	hPARP2-1 −1–373 −DNA	hPARP2-1 −1–373 +DNA	hPARP2-1 +1–373 +DNA	hPARP2-1 +1–373 +DNA
V_{max} ($\mu mol \times min^{-1} \times mg^{-1}$)	0.2–2.4	NC	NC	0.114 ± 0.010	0.488 ± 0.026
K_m (μM)	59–278	NC	NC	1111 ± 127.7	140.8 ± 19.72
K_{cat} (s^{-1})	0.41	NC	NC	0.121 ± 0.011	0.521 ± 0.028
K_{cat}/K_m ($s^{-1} \times mM^{-1}$)	1.475–6.949	NC	NC	0.109 ± 0.022	3.696 ± 0.715

NC: not calculable (product levels below detection limit).
[a]Values as reported in the literature.

respectively) (Supplementary Figure 3A). A molar ratio of 1:5 (wt:mutant) severely reduced PAR formation (Figure 5B), suggesting that the different proteins are indeed able to form dimers and to regulate each other.

We showed earlier that only the full-length DBD was able to stimulate chimera PARP2-1 (Figure 4). This finding suggests that disruption of the DBD of PARP1 by deleting one of the DBD sub-domains would render the protein inactive due to lost activation of the CAT. Deletion of the regions containing zinc finger FI (ΔFI, aa 1–111) or zinc-binding domain FIII (ΔFIII, aa 279–333) indeed rendered PARP1 inactive, while a mutant lacking FII (ΔFII, aa 117–201) was still able to synthesize PAR as examined by western blot analysis (Figure 5A and C). Thus, the presence of FI and FIII is required for enzymatic activity, while neither FII nor the spacing between FI and FIII seem to be critical for PAR formation. Surprisingly, co-incubation of the two inactive mutants ΔFI and ΔFIII fully restored activity, suggesting that the two proteins can interact and that the lack of the critical domains containing FI and FIII could be intermolecularly complemented to form a functional active dimer (Figure 5D). In line with this finding, the mutants ΔFI and ΔFIII could also be complemented by co-incubation with the catalytically inactive PARP1 mutants E988K and M890V/D899N or with the DBD alone (Figure 5E).

The WGR domain is vital for enzymatic activity

We showed that the WGR domain is required for enzymatic activity of PARP1 (Figure 4). A PARP1 deletion mutant lacking the WGR domain (ΔWGR, aa 525–656) was indeed not able to generate PAR (Figure 5F). Similarly, a PARP2 deletion mutant lacking the WGR domain was also inactive (data not shown), suggesting that the so far uncharacterized WGR domain of PARP family members is absolutely required for the enzymatic activity. Importantly, the PARP1 ΔWGR mutant could functionally complement the two PARP1 mutants ΔFI and ΔFIII by providing its DBD (Figure 5G). Co-incubation of the PARP1 ΔWGR mutant with the catalytically inactive PARP1 mutants E988K and M890V/D899N, both possessing a functional WGR, however, did not restore enzymatic activity (Figure 5G). Thus, the PARP1 DBD and the CAT can be regarded as independent and flexible protein units in a catalytic dimer, whereas the WGR domain is functionally tightly associated with and required for the activation of the CAT.

Glutamic acid residues in the AD of PARP1 are not modified

It is widely believed that multiple glutamic acid residues within the AD of PARP1 serve as acceptor sites for the covalent attachment of PAR (16). To our knowledge, however, this assumption has so far not been confirmed by amino acid substitutions. Therefore, we decided to test whether glutamic acid residues within the AD of PARP1 are required for the catalytic activity of the enzyme and function as acceptors for PAR. First, we deleted the BRCT domain as part of the AD (Figure 6A). A PARP1 ΔBRCT mutant was as active as its wild-type counterpart with regard to auto-modification (Figure 6B, first four lanes) and PAR formation (Supplementary Figure 5A, left panel). Next, in the context of the PARP1 ΔBRCT mutant, we additionally mutated all eight glutamic acid residues in the remaining auto-modification loop between amino acids 484 and 557 to glutamine (ΔBRCT/E) (Figure 6A). Surprisingly, these substitutions also did not reduce auto-modification (Figure 6B, last two lanes) or PAR formation (Supplementary Figure 5A, right panel). These results strongly indicate that glutamic acid residues within the AD of PARP1 are not required for enzymatic activity and are unlikely to serve as acceptors for PAR.

Lysine residues are acceptor sites in PARP1

In contrast to the deletion of the BRCT domain, deletion of the remaining amino acids in the AD of PARP1 (ΔAc, aa 466–525) (Figure 6A), a region previously reported to be acetylated (26), resulted in severely impaired auto-modification (Figure 6C) and reduced PAR formation (Supplementary Figure 5B), suggesting that acceptor sites are localized in this region of PARP1. As PAR levels generated by PARP1 ΔAc were decreased but did not drop completely, additional PAR acceptor sites are likely to exist in other domains of PARP1. Trans-poly(ADP-ribosyl)ation of different fragments of PARP1 by wild-type PARP1 indeed confirmed that not only the AD but also a fragment containing amino acid 1–214 is modified (Supplementary Figure 5C).

ADP-ribose has earlier been described to be a potent histone glycation and glycoxidation agent *in vitro*, leading to the formation of ketoamine glycation conjugates (30).

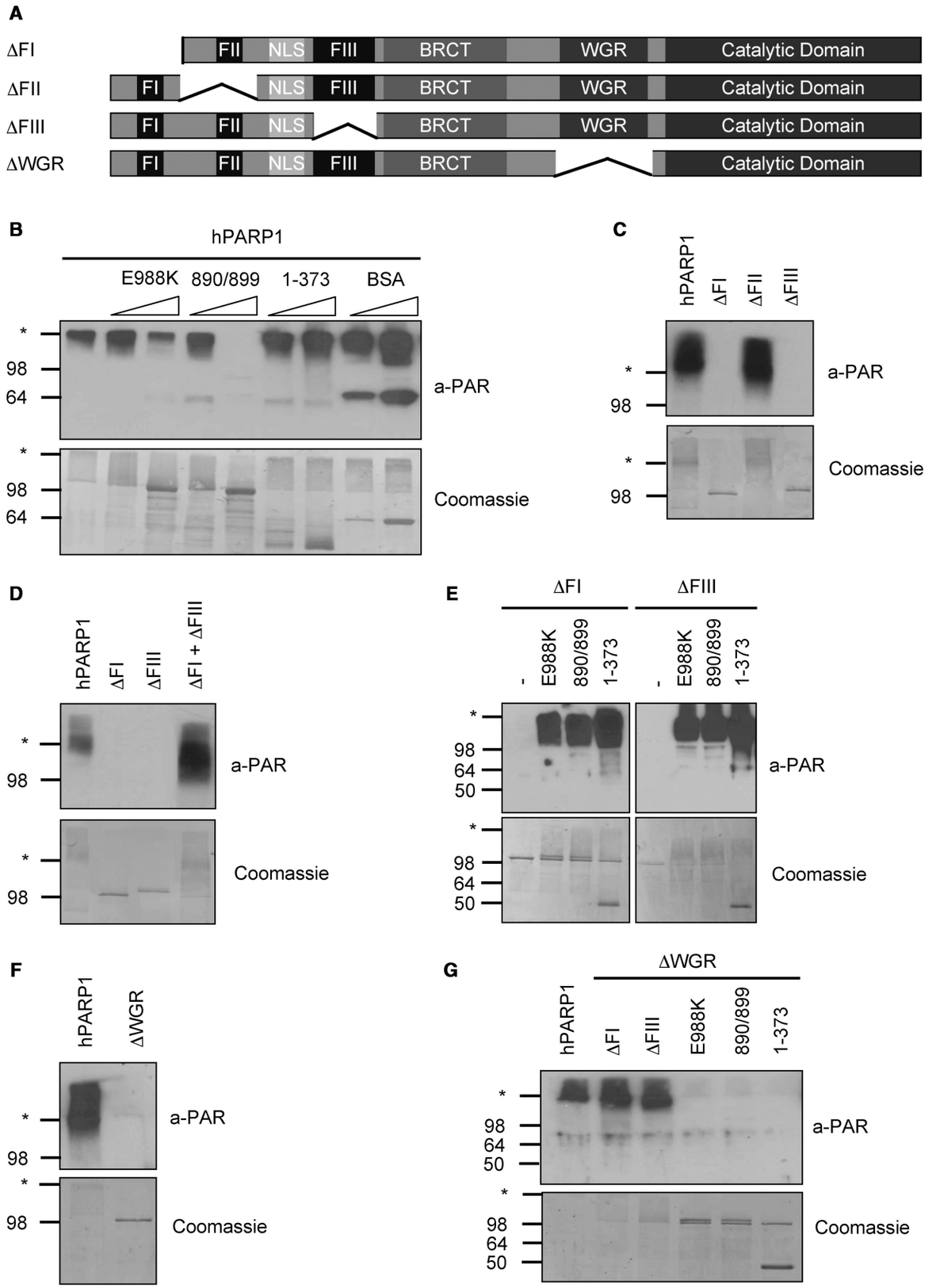

Figure 5. PARP1 forms a catalytic dimer which requires at least one functional FI and FIII domain for activity. (**A**) Domain organization of the PARP1 deletion mutants used for this figure. (**B**) PAR formation by PARP1 when co-incubated with the indicated inactive proteins or fragments at a molar ratio of 1:1 or 1:5. According to the manufacturer, the anti-PAR antibody LP96-10 cross reacts with bovine serum albumin (BSA) (band at

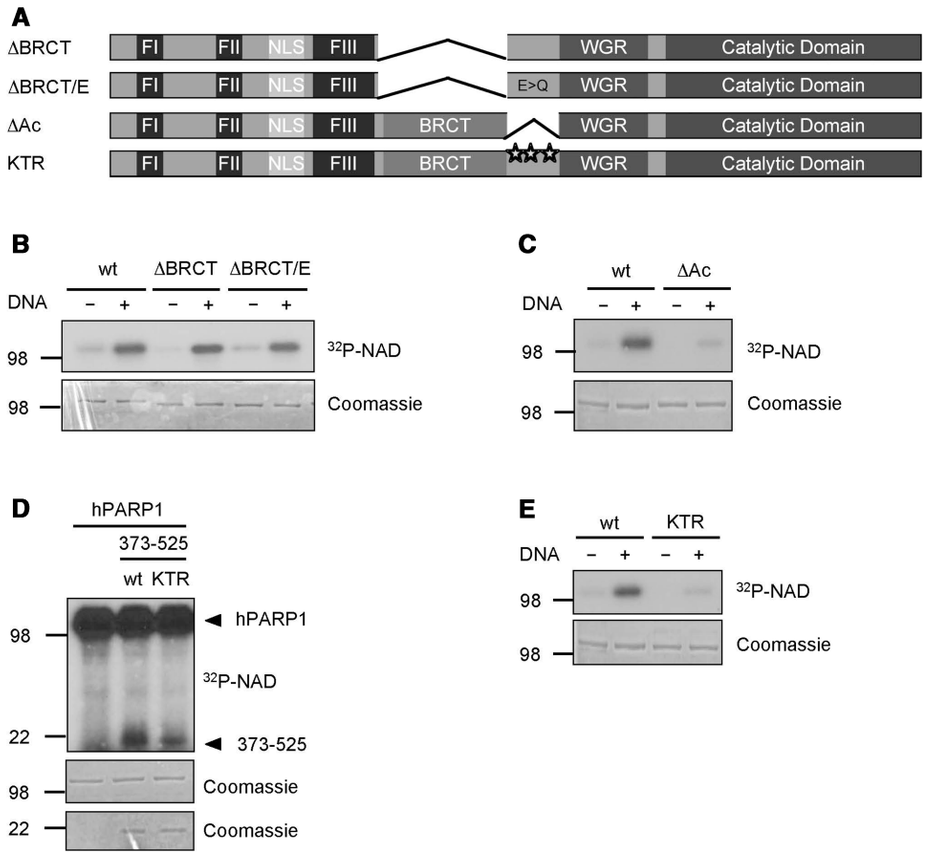

Figure 6. Lysine residues within the AD of PARP1 are target sites for poly(ADP-ribosyl)ation. (**A**) Domain organization of the PARP1 mutants used for this figure. (**B**) Auto-modification of the indicated PARP1 mutants lacking either the BRCT domain (ΔBRCT) or the BRCT domain and carrying substitutions for all glutamic acid residues in the remaining stretch of the AD (ΔBRCT/E). (**C**) Auto-modification of a PARP1 deletion mutant lacking aa 466–525 (ΔAc), a region that was previously shown to be acetylated. (**D**) Trans-poly(ADP-ribosyl)ation of the AD from amino acid 373–525 by PARP1. KTR, K498/521/524R. (**E**) Auto-modification of a PARP1 K498/521/524R mutant. Molecular size markers in kilo Daltons are indicated.

When we analyzed the chemical linkage stability of auto-modified PARP1, we found that it was stable up to pH 10 (but not at pH 13.5) and that incubation with 1 M hydroxylamine at pH 7 for 30 min at 30°C or 60°C did not release the modification (Supplementary Figure 5D). This observation suggests that the protein-ADP-ribose linkage indeed might occur on lysines. To exclude that the investigated auto-modification of PARP1 was due to traces of ADP-ribose within the provided NAD, the inactive PARP1 mutant M890V/D899N was incubated with radioactive NAD. Only upon long exposure a faint labeling of PARP1 M890V/D899N was observed while PARP1 E988K was able, as described earlier, to modify itself (Supplementary Figure 5E), confirming that the observed auto-modification of PARP1 was due to its enzymatic activity.

Next, we analyzed the trans-poly(ADP-ribosyl)ation of a fragment comprising the AD (aa 373–525) of PARP1 by full-length PARP1. Although auto-modification of PARP1 was much more efficient than trans-poly(ADP-ribosyl)ation, specific labeling of the AD fragment was observed (Figure 6D, second lane). In order to identify individual lysine residues within the AD which serve as acceptor sites for PAR, we analyzed trans-poly(ADP-ribosyl)ation of the AD fragment containing three lysine to arginine substitutions (K498, K521 and K524, called KTR). These sites were previously reported to be targets for acetylation (26). Modification of the 373–525 KTR fragment by full-length PARP1 was reduced as compared to 373–525 wt (Figure 6D, third lane). Since trans-poly(ADP-ribosyl)ation of a protein fragment might lead, due to structural constrains, to unspecific modification of amino acids and might thus not be comparable to modification of the full-length protein, we generated a full-length PARP1 mutant which contains the three lysine to arginine substitutions at position 498, 521 and 524.

around 64 kDa). (**C**) PAR formation by DBD deletion mutants ΔFI, ΔFII and ΔFIII. (**D**) PAR formation by a combination of the two DBD deletion mutants ΔFI and ΔFIII. (**E**) PAR formation by the DBD deletion mutants ΔFI and ΔFIII when they were co-incubated with catalytically inactive PARP1 mutants or with fragment 1–373. (**F**) PAR formation by PARP1 lacking the WGR domain. (**G**) PAR formation by PARP1 ΔWGR in combination with DBD deletion mutants, catalytically inactive PARP1 mutants or with fragment 1–373. Molecular size markers in kilo Daltons and the border between stacking and separating gel (*asterisk*) are indicated.

Importantly, this mutant showed strongly reduced auto-modification, very much comparable to the levels observed for the PARP1 ΔAc mutant (Figure 6E). Overall, these experiments provide evidence that not glutamic acid residues but instead at least three lysine residues within the auto-modification loop (aa 466–525) and additional residues within the first 214 amino acids of PARP1 are target sites for enzymatic auto-ADP-ribosylation.

DISCUSSION

In this study we analyzed the poly(ADP-ribosyl)ation capacity of PARP1 and the closely related proteins PARP2 and PARP3 under standardized reaction conditions and investigated the molecular mechanism of PAR formation. Human PARP1 and PARP2 were able to auto-modify themselves and generate PAR, although to different levels. Neither polymer formation nor auto-modification was observed for PARP3 under the tested conditions.

PARP1 deletion mutants and fusion proteins had been successfully employed before to study different aspects of poly(ADP-ribosyl)ation (31,32). Here, we have generated PARP family chimera to analyze the molecular mechanism of PAR formation. The PARP chimera revealed that the WGR/CAT domains of PARP1, PARP2 and PARP3 tightly cooperate with their corresponding amino-terminal domains. Closer examination of PARP1 revealed that FI, FIII and the WGR/CAT domain of PARP1 are required and sufficient for PAR formation. FII and the BRCT domain, however, were not essential for the enzymatic activity. The DBD interacted directly with the CAT domain of PARP1. DBD bound to DNA increased V_{max} and reduced the K_m of the CAT for NAD. We also provide evidence that PARP1 forms a catalytic dimer in which lack of either FI or FIII could be functionally complemented by a protein containing these domains. Finally, we identified three lysine residues within the AD and additionally the first 214 amino acids of the DBD as target sites for enzymatic covalent auto-poly(ADP-ribosyl)ation by PARP1.

We employed three different methods to assess poly(ADP-ribosyl)ation. First, ^{3}H-NAD at a concentration of 400 μM was used to measure TCA-precipitable polymer formation in time course experiments. Second, ^{32}P-NAD at a concentration of only 100 nM was used to measure auto-modification after short incubation periods (10 s). This approach resulted in distinct bands corresponding to the modified protein and most likely representing mono(ADP-ribosyl)ation or short oligomers of ADP-ribose attached to the labeled protein. Third, unlabeled NAD at a concentration of 400 μM was used to measure PAR formation detected by western blot. The anti-PAR antibody typically detected high molecular weight polymers, most of which remained as a smear at the top of the separating gel or even in the stacking gel. This approach was not very suitable to make quantitative statements but could be readily applied to analyze whether a protein was active or not.

Human PARP3 was previously described by Augustin *et al*. to be an active enzyme, as detected by autoradiography after 15 min incubation with 10 μM ^{32}P-NAD (19). Augustin and co-workers did not, however, compare the activity of PARP3 to that of PARP1 or any other PARP family member under these conditions. We analyzed PARP3 in comparison to PARP1 and PARP2 under standardized reaction conditions and could not observe any activity for this protein. However, when we applied the conditions provided by Augustin *et al*. to measure PARP3 activity by autoradiography, we could also observe PARP3 auto-modification (data not shown), suggesting that the protein possesses some degree of activity under certain well-defined conditions. Further investigations are needed to analyze the extent of PAR formation by PARP3 as well as its physiological relevance.

The DBD of PARP1 interacted in a coordinated and DNA-dependent manner with the CAT domain of PARP1, but not with that of PARP2 or PARP3. Thus, despite the high level of structural similarity between the CATs of PARP1, PARP2 and PARP3, these domains cannot compensate for each other and may possess unanticipated intrinsic regulatory functions. Since the PARP1 DBD is not or only partially present in other PARP family members, the newly identified intra-molecular interaction might provide a promising surface for the development of PARP1 specific inhibitors.

In our study, several enzymatic dead mutants with deletions in the DBD could be functionally complemented by another inactive PARP1 mutant containing the missing domain, thus implicating that PARP1 is forming a dimer for poly(ADP-ribosyl)ation. The existence of catalytically active protein dimers in which each monomer is lacking a domain required for enzymatic activity was surprising and suggests that PARP1 is a highly flexible molecule with rather loose domain architecture.

Consistent with earlier reports (33,34) our results showed that zinc finger FI is absolutely required for the DNA-dependent activation of the protein, whereas zinc finger FII is dispensable. Zinc finger FII may, however, determine the binding specificity for DNA single strand breaks as suggested previously by Gradwohl *et al*. (35). Our data revealed that the recently discovered zinc-binding motif FIII is essential for the interaction of the DBD with the CAT and thus also for the activation of the enzyme. Furthermore, the so far uncharacterized WGR domain is an indispensable prerequisite for PAR formation, although this domain is not necessary for the interaction between the DBD and the CAT.

The interaction between the DBD bound to DNA and the CAT domain increased the maximum reaction velocity V_{max} by a factor of four and reduced the K_m for NAD roughly from 1 mM to 140 μM (Table 1). The reaction efficiency K_{cat}/K_m was thereby increased by a factor of more than 30. The total cellular NAD concentration was previously estimated to be around 350 μM (36). Zhang *et al*. argued that NAD cofactors should readily pass through nuclear pores, which would suggest that cytoplasmic NAD levels reflect nuclear NAD concentrations (37). The same group estimated the free nuclear NAD concentration to be around 70 μM (38). Despite this uncertainty

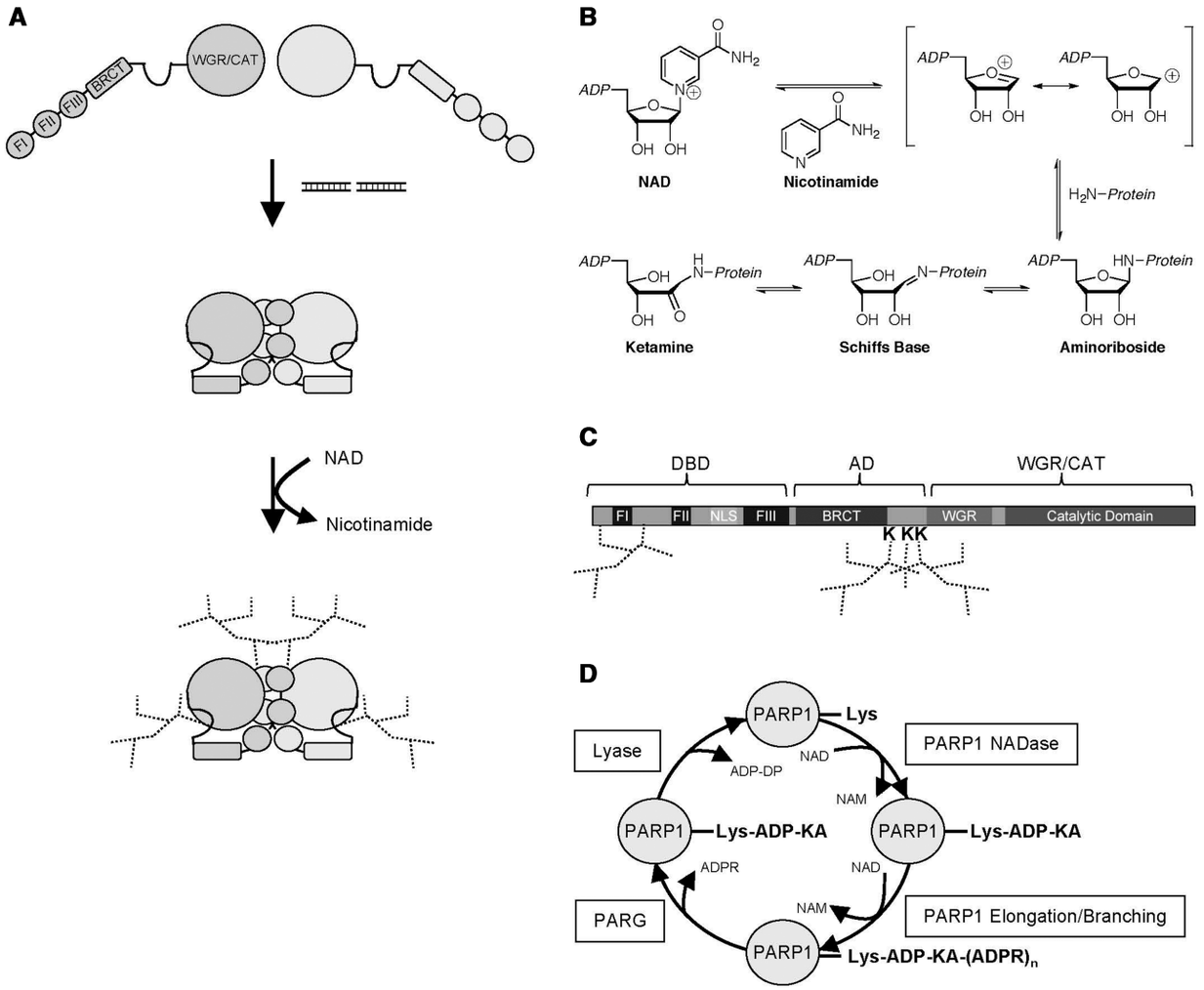

Figure 7. Model for PARP1 activation and ADP-ribosylation of lysine residues. (**A**) Model for the sequential activation and regulation of PARP1. The DNA-dependent interaction between the DBD and the WGR/CAT induces a state of high substrate affinity and high turnover rate in PARP1. Subsequently, acceptor amino acids in the auto-modification loop as well as in the DBD are poly(ADP-ribosyl)ated. (**B**) Proposed reaction mechanism for NADase-dependent auto-ADP-ribosylation of lysine residues by PARP1 via Schiff base formation. (**C**) Scheme depicting the identified PAR acceptor lysine residues in PARP1. (**D**) A revised view of ADP-ribose metabolism. PARP1 catalyzes lysine mono(ADP-ribosyl)ation via its NADase activity and subsequently PAR chain elongation. PARG cleaves glycosidic ribose–ribose bonds to generate PARP1-Lys-ADP-KA, which is then substrate for an ADP-ribosyl protein lyase. See discussion for details. NAM, nicotinamide; Lys, lysine; ADP-KA, ADP-ketamine; ADPR, ADP-ribose; ADP-DP, ADP-3″-deoxypentose-2″-ulose.

in the estimation of free nuclear NAD concentrations, we believe that increasing the affinity of PARP1 for NAD by binding to DNA double strand breaks might be an important regulatory step to allow PAR formation at physiological NAD concentrations. Release of PARP1 from DNA would consequently reduce the affinity of PARP1 for NAD and terminate PAR formation. Importantly, the nuclear concentration of NAD can be modulated by NMN adenylyl transferase 1 (NMNAT-1), which catalyzes the final step of NAD biosynthesis. A recent study revealed that NMNAT-1 is able to interact with and stimulate PARP1 (39). It is thus tempting to speculate that PARP1 activation by its binding to DNA strand breaks is supported by the localized action of NMNAT-1.

Our results suggest that activation of PARP1 occurs in defined sequential steps (Figure 7A). First, the DBD binds to certain damages within the DNA. This enhances the interaction between the DBD and the CAT domain. As a consequence, minor structural rearrangements within the catalytic cleft occur, resulting in an increased affinity for NAD. Increasing substrate affinity and additionally substrate turnover rates then allows for high reaction efficiency and very rapid automodification at distinct lysine residues followed by PAR chain elongation. An analogous model can be envisioned for the protein chimera PARP2-1, which is activated by the PARP1 DBD in the presence of double strand breaks mimicking DNA (Supplementary Figure 6A).

The assumption that PARP1 is modified at glutamic acid residues was based mainly on the chemical stability of the ADP-ribose-PARP1 linkage, which was very heterogeneous but in part of a similar type as carboxyl esters in mono-ADP-riboslyated histones isolated from cells (23). The presented mutation analysis studies revealed that neither deletion of all glutamic acid residues in the BRCT domain (aa 385–476, containing nine glutamic acid residues) nor additional mutation of the remaining glutamic acid residues to glutamine in the AD (aa 477–557, containing eight glutamic acid residues) affected auto-modification or PAR formation and thus provide strong evidence that these amino acids in the AD are not the acceptor sites for poly(ADP-ribosyl)ation. Interestingly, mutation of the three lysines K498, K521 and K524 in the AD of PARP1 to arginines strongly reduced the auto-modification of the enzyme, suggesting that these residues in fact are acceptors for PAR. A longer exposure of the gel revealed a weak labeling of PARP1 KTR (data not shown) and it may well be that additional lysine residues serve as acceptor sites in this domain. Furthermore, acceptor sites can also be expected in the DBD of PARP1. Whether these sites are also lysine residues or whether outside the AD other amino acids serve as PAR acceptors is currently not known.

Modification of proteins by ADP-ribose can be characterized according to their chemical properties. ADP-ribosylated lysine residues were described to be stable in the presence of 1 M hydroxylamine at pH 7, while chemically modified glutamic and aspartic acid residues would rapidly release the ADP-ribose moiety (40,41). Our chemical analysis of modified PARP1 revealed that the observed linkage most likely corresponds to the glycation linkage described above. Thus we propose that ADP-ribosylation of PARP1 is catalyzed by its NADase activity, which subsequently allows modification of lysine residues positioned close to the catalytic active site to Lys-ADP-ribose ketamine (Figure 7B, C and D). This moiety could then serve as acceptor for the elongation reaction, which is catalyzed by glutamic acid residue E988 in human PARP1. We are currently investigating whether other ADP-ribose acceptor proteins are modified by PARP1 in the same manner.

To date two enzymes, poly(ADP-ribose) glycohaydrolase (PARG) and ADP-ribosyl protein Lyase, have been described to be involved in PAR catabolism (42,43). While PARG possesses both exo- and endoglycosidic activities, the Lyase was described to cleave the bond between proteins and mono(ADP-ribose). ADP-ribosylation of lysines creates a chemical bond, which is not a substrate for PARG, which cleaves the ribose–ribose bonds. Breaking a lysine-ADP-ribose linkage would instead require the activity of a Lyase (Figure 7D). Alternatively, the last ADP-ribose moieties might remain on PARP1 to serve as elongation sites for the next round of PAR formation or to mark the chromatin to memorize the location of previous DNA damage repair.

Lysine residues K498, K521 and K524 were previously identified as targets for acetylation by p300 and P300/CBP-associated factor (PCAF) in a stimulus-dependent manner (26). Remarkably, simple addition of PCAF reduced poly(ADP-ribosyl)ation by PARP1 (unpublished observation), suggesting that the interaction domain of PARP1 with PCAF is overlapping with the ADP-ribose acceptor sites. Furthermore, we recently showed that acetylation of PARP-2 strongly reduced the enzymatic activity (44). Already more than 20 years ago, a possible interrelation between poly(ADP-ribosyl)ation reactions and post-translational protein acetylation had been discussed (45,46). Our finding that acetylation of lysine residues interferes with ADP-ribosylation supports this idea and points at an interesting crosstalk between acetylation of and ADP-ribosylation by PARP family members. This crosstalk hypothesis is further strengthened by the finding that the enzymatic activity of PARP1 is not required for the function as transcriptional co-activator of NF-κB, a role which requires acetylation of PARP1 (47).

During apoptosis, PARP-1 is cleaved by different caspases to generate 89-kDa and 24-kDa fragments, a well-characterized hallmark of apoptosis. The data shown provide a functional explanation for the observed inactivation of PARP1 upon caspase cleavage, as this cleavage is separating FI and FII (aa 1–214) from FIII (aa 214–373), thus no longer allowing the DBD to interact as an intact polypeptide with the CAT domain for subsequent activation.

Our chemical and mutational analyses provide evidence that lysine residues are acceptor sites for auto-modification by PARP1 *in vitro* (Figure 6B). As PARP1 is the main acceptor protein for poly(ADP-ribosyl)ation *in vivo* (48,49), our findings are most likely also relevant *in vivo*. Confirming acceptor sites *in vivo*, however, is very difficult for different reasons. First, mutations within the DBD to eliminate the acceptor sites in this region and to allow only the analysis of the three lysine residues in the auto-modification domain would affect the activation of PARP1 by DNA. Second, PARP1 is known to be modified by PARP2 and possibly by other PARP family members. Whether theses proteins are modifying PARP1 also at the auto-modification sites or at other residues is currently not known. In any case, however, this crosstalk would interfere with *in vivo* analysis of PARP1 auto-modification.

In conclusion, we propose that PARP1 forms a catalytic dimer that allows the interaction of the DNA-binding domain with the CAT to modify distinct lysine residues as ADP-ribose acceptor sites in the AD as well as additional acceptor sites in the DNA-binding domain. These insights will allow further investigations to elucidate the biological functions of PARP1 and its enzymatic activity.

SUPPLEMENTARY DATA

Supplementary Data are available at NAR Online.

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Conflict of interest statement. None declared.

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Supplementary Figure Legends

Supplementary Figure 1:

(A) Time-dependent PAR formation by PARP1 in the absence or presence of DNA as revealed by western blot. (B) Time-dependent PAR formation by PARP1 in the absence or presence of DNA as revealed by vacuum slot blot. (C) PAR formation by PARP1, PARP2 and PARP3. (D) PAR formation by PARP1, chimera PARP1-2 and chimera PARP1-3. (E) PAR formation of chimera PARP2-1, chimera PARP3-1 and PARPs-1. PARP1 served as control (ctr.). Molecular size markers in kDa and the border between stacking and separating gel (asterisk) are indicated.

Supplementary Figure 2:

(A) Overlay of the crystal structures available for the catalytic domains of chicken PARP1 (PDB: 1A26), mouse PARP2 (PDB: 1GS0) and human PARP3 (PDB: 2PA9). PDB files were obtained from RCSB PDB (www.rcsb.org/pdb/home/home.do), the alignment was performed using the Magic Fit function of the Swiss-PDBViewer with the catalytic domain of chicken PARP1 as template. Yellow: PARP1, blue: PARP2, green: PARP3. (B) Sequence alignment of the conserved carboxyl-termini of PARP1, PARP2 and PARP3. The alignment was performed using Clustal2W (www.ebi.ac.uk/Tools/clustalw2/index.html). The conserved residues VDP at position 533 to 535 in hPARP1 used to generate the different PARP family chimera are marked.

Supplementary Figure 3:

(A) PARP1 E988K and PARP1 M890V/D899N are enzymatically inactive as revealed by western blot. (B) The stimulation of PARP2-1 by fragment 1-373 is resistant to high salt conditions (left panel). The stimulation of PARP2-1 by fragment 1-373 is not due to a PAR carrier effect of fragment 1-373 (middle panel). * 1-373 was added after the reaction. The stimulation of PARP1 373-1014 by fragment 1-373 is resistant to high salt conditions (right panel). Molecular size markers in kDa and the border between stacking and separating gel (asterisk) are indicated.

Supplementary Figure 4:

(A) PAR formation after long incubation of PARP2-1, PARP3-1, PARPs-1 and PARP 656-1014 in the absence or presence of DNA and PARP1 fragment 1-373 as revealed by western blot (60 minutes incubation, upper panel), coomassie staining (60

minutes incubation, middle panel) or auto-modifcation as detected by autoradiography (5 minutes incubation, lower panel). Molecular size markers in kDa and the border between stacking and separating gel (asterisk) are indicated. (B) PAR formation after 5 minutes incubation by PARP family members and PARP chimera in the absence or presence of PARP1 fragment 1-373 as revealed by silver staining. Equal molar amounts of proteins were used. (C) Time course of PAR formation by PARP2, PARP3, PARP1-2 and PARP1-3 in the absence or presence of fragment 1-373. Reactions were performed in triplicates, error bars represent standard deviations.

Supplementary Figure 5:

(A) PAR formation by the PARP1 mutants ΔBRCT and ΔBRCT/E. (B) PAR formation by the PARP1 mutant ΔAc. (C) Trans-poly(ADP-ribosyl)ation of different PARP1 fragments by full-length PARP1. 1, aa 1-214; 2, aa 215-373; 3, aa 373-525; 4, aa 525-656; 5, aa 656-1014. (D) Stability of the PARP1-ADP-ribose linkage under different pH conditions. Auto-modified PARP1 was incubated for 30 minutes at 60°C (top) or 30°C (bottom) under the indicated conditions and subsequently subjected to SDS-PAGE and detection by autoradiography. Tris-HCl pH 8.0 served as control. (E) Auto-modification of PARP1 mutants. * PARP inhibitor 3-aminobenzamide (3-AB) was added after the reaction. A long exposure was required to detect the shown levels of auto-modification. Molecular size markers in kDa and the border between stacking and separating gel (asterisk) are indicated.

Supplementary Figure 6:

(A) Model for the sequential activation and regulation of chimera PARP2-1 by the PARP1 DBD bound to double strand break mimicking DNA. The DNA-dependent interaction between the PARP1 DBD and the PARP1 WGR/CAT induces a state of high substrate affinity and high turnover rate. Subsequently, acceptor amino acids most likely situated in the amino-terminal region of chimera PARP2-1 and in the PARP1 DBD are poly(ADP-ribosyl)ated. (B) Scheme depicting PAR acceptor lysine residues in chimera PARP2-1 in accordance with our previous finding, that lysines 36 and 37 of mPARP2 are targets for auto-ADP-ribosylation (1).

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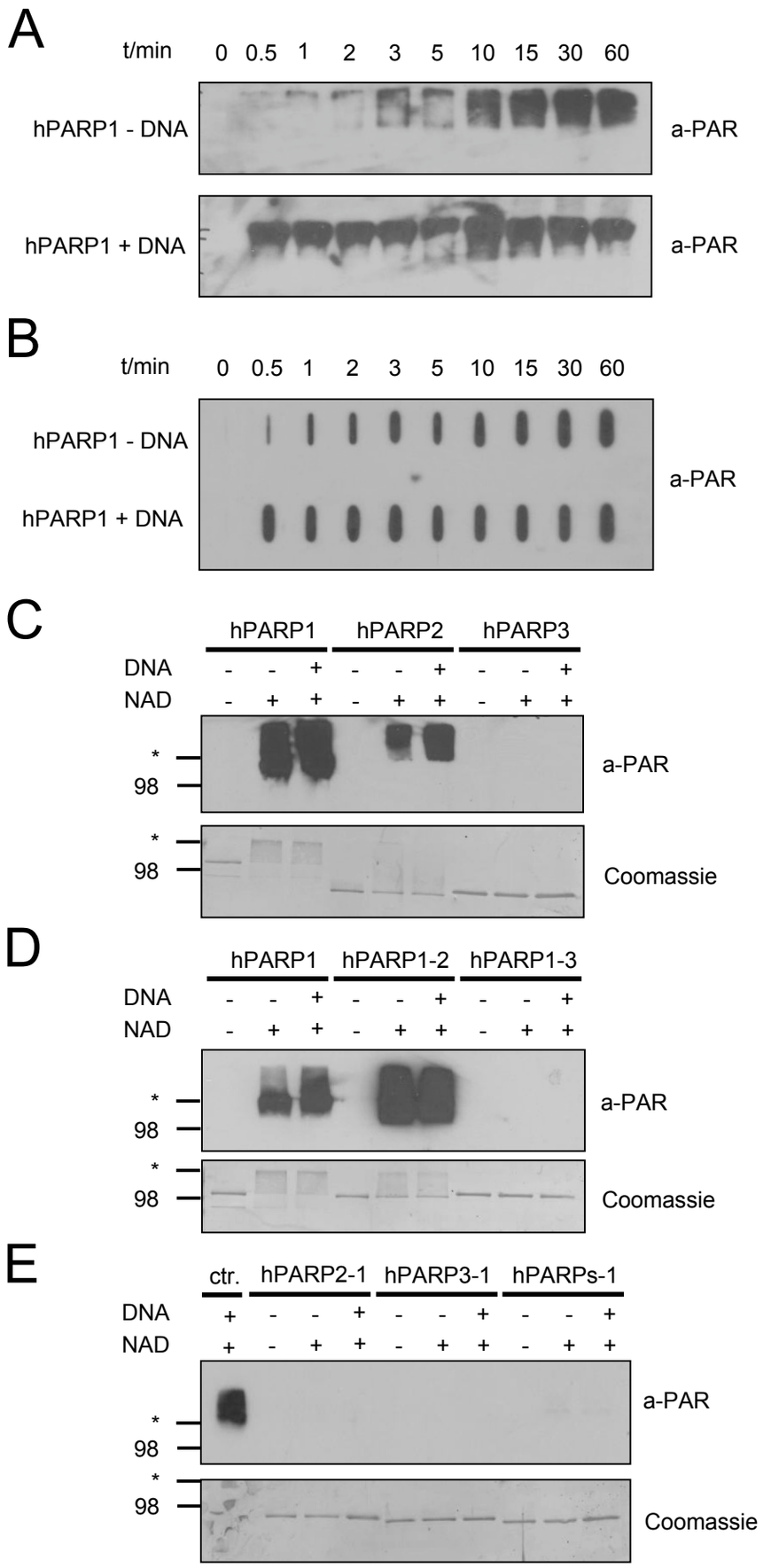
A
t/min
0 0.5 1 2 3 5 10 15 30 60
hPARP1 - DNA
hPARP1 + DNA
a-PAR
B
t/min
0 0.5 1 2 3 5 10 15 30 60
hPARP1 - DNA
hPARP1 + DNA
a-PAR
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hPARP1 hPARP2 hPARP3
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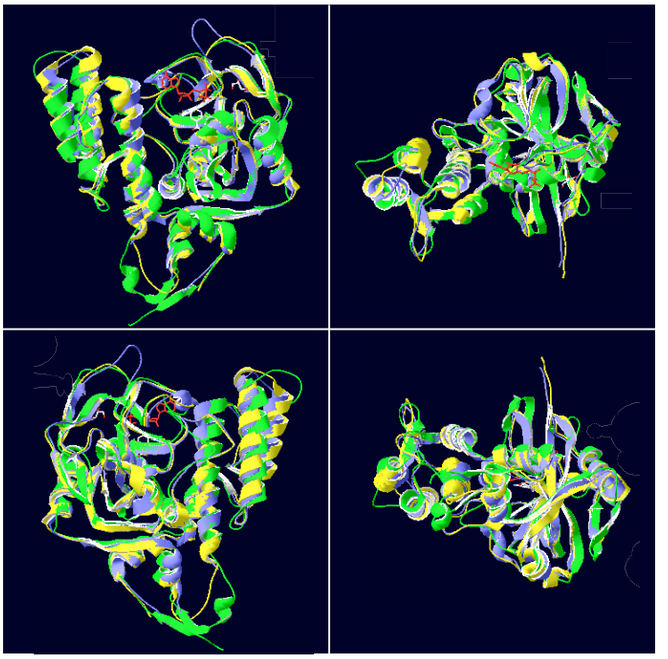

B

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hPARP1   WGAEVKAEPVEVVAPRGKSGAALSKKSKGQVKEEGINKSEKRMKLTLKG-GAAVDPDSGL 539
hPARP2   --------------------VAGGKANKDRTEDK---QDESVKALLLKG-KAPVDPECTA 87
hPARP3   ------------------------GRQAGREEDPFRSTAEALKAIPAEKRIIRVDPTCPL 54
                                 . .: ::      *    :  :     ***  .

hPARP1   EH-SAHVLEKGGKVFSATLGLVDIVKGTNSYYKLQLLEDDKENRYWIFRSWGRVGTVIGS 598
hPARP2   KVGKAHVYCEGNDVYDVMLNQTNLQFNNNKYYLIQLLEDDAQRNFSVWMRWGRVGKMGQH 147
hPARP3   SSNPGTQVYED---YNCTLNQTNIENNNNKFYIIQLLQD-SNRFFTCWNRWGRVGEVGQ- 109
         .   .    :.   :.  *. .::  ..*.:* :***:*  :. :  :  ***** :

hPARP1   NKLEQMPSKEDAIEHFMKLYEEKTGNAWHSKN-FTKYPKKFYPLEIDYG---QDEEAVKK 654
hPARP2   SLVACSGNLNKAKEIFQKKFLDKTKNNWEDREKFEKVPGKYDMLQMDYATNTQDEEETKK 207
hPARP3   SKINHFTRLEDAKKDFEKKFREKTKNNWAERDHFVSHPGKYTLIEVQAEDEAQEAVVKVD 169
         . :      :.* : * * : :** * * .:: * . * *:  ::::     *:     .

hPARP1   ---LTVNPG--TKSKLPKPVQDLIKMIFDVESMKKAMVEYEIDLQKMPLGKLSKRQIQAA 709
hPARP2   EESLKSPLK--PESQLDLRVQELIKLICNVQAMEEMMMEMKYNTKKAPLGKLTVAQIKAG 265
hPARP3   RGPVRTVTKRVQPCSLDPATQKLITNIFSKEMFKNTMALMDLDVKKMPLGKLSKQQIARG 229
            :         ..*   .*.**. * . : ::: *   . : :* *****:  **  .

hPARP1   YSILSEVQQAVS-QGSSDSQILDLSNRFYTLIPHDFGMKKPPLLNNADSVQAKAEMLDNL 768
hPARP2   YQSLKKIEDCIR-AGQHGRALMEACNEFYTRIPHDFGLRTPPLIRTQKELSEKIQLLEAL 324
hPARP3   FEALEALEEALKGPTDGGQSLEELSSHFYTVIPHNFGHSQPPPINSPELLQAKKDMLLVL 289
         :. *. :::.:    . .  : : ...*** ***:**   ** :.. . :. * ::*  *

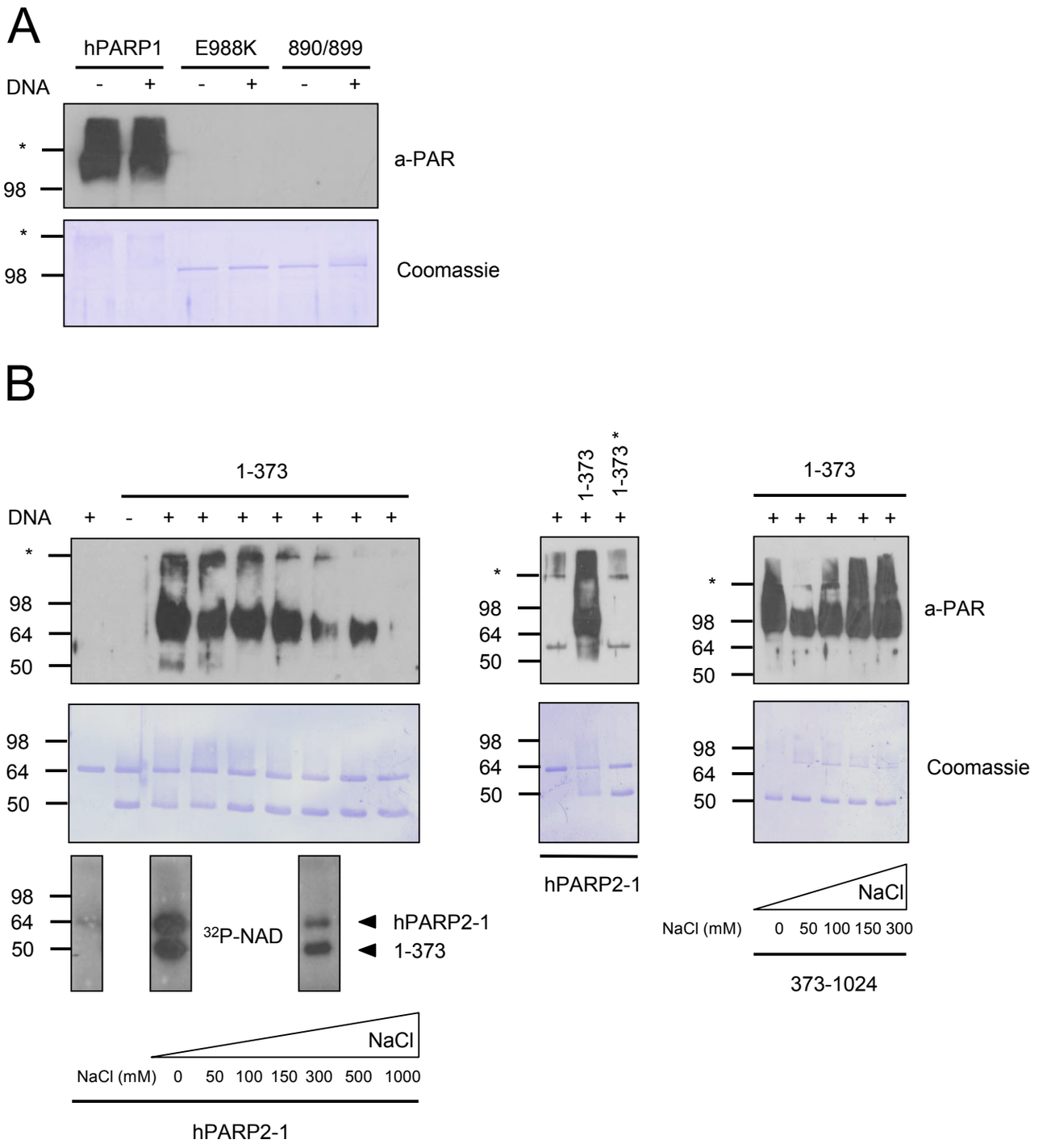
hPARP1   LDIEVAYSLLRGGSDD----SSKDPIDVNYEKLKTDIKVVDRDSEEAEIIRKYVKNTHAT 824
hPARP2   GDIEIAIKLVKTELQ-----SPEHPLDQHYRNLHCALRPLDHESYEFKVISQYLQSTHAP 379
hPARP3   ADIELAQALQAVSEQEKTVEEVPHPLDRDYQLLKCQLQLLDSGAPEYKVIQTYLEQTGSN 349
          ***:*  *     :     .  .*:* .*. *:  :: ::*  : * ::*  *::.* :

hPARP1   THNAYDLEVIDIFKIEREGECQRYKPFKQLHNRRLLWHGSRTTNFAGILSQGLRIAPPEA 884
hPARP2   THSDYTMTLLDLFEVEKDGEKEAFR--EDLHNRMLLWHGSRMSNWVGILSHGLRIAPPEA 437
hPARP3   HR---CPTLQHIWKVNQEGEEDRFQAHSKLGNRKLLWHGTNMAVVAAILTSGLRIMP--- 403
          :      : .::::::::** : ::  ..* ** *****:. :  ..**: **** *

hPARP1   PVTGYMFGKGIYFADMVSKSANYCHTSQG--DPIGLILLGEVALGNMYELKHASHISK-L 941
hPARP2   PITGYMFGKGIYFADMSSKSANYCFASRL--KNTGLLLLSEVALGQCNELLEANPKAEGL 495
hPARP3   -HSGGRVGKGIYFASENSKSAGYVIGMKCGAHHVGYMFLGEVALGREHHINTDNPSLKSP 462
           :*  .*******.  ****.*    :   .  * ::*.*****.  .:   .   :

hPARP1   PKGKHSVKGLGKTTPDP--SANISLDGVDVPLGTGISSGVNDT---SLLYNEYIVYDIAQ 996
hPARP2   LQGKHSTKGLGKMAPSS--AHFVTLNGSTVPLGPASDTGILNPDGYTLNYNEYIVYNPNQ 553
hPARP3   PPGFDSVIARGHTEPDPTQDTELELDGQQVVVPQGQPVPCPEFSSSTFSQSEYLIYQESQ 522
           * .*. . *:  *..     : *:*  * :  .      :    ::  .**::*:  *

hPARP1   VNLKYLLKLKFNFKTSLW 1014
hPARP2   VRMRYLLKVQFNFLQ-LW 570
hPARP3   CRLRYLLEVHL------- 533
          .::***::::
```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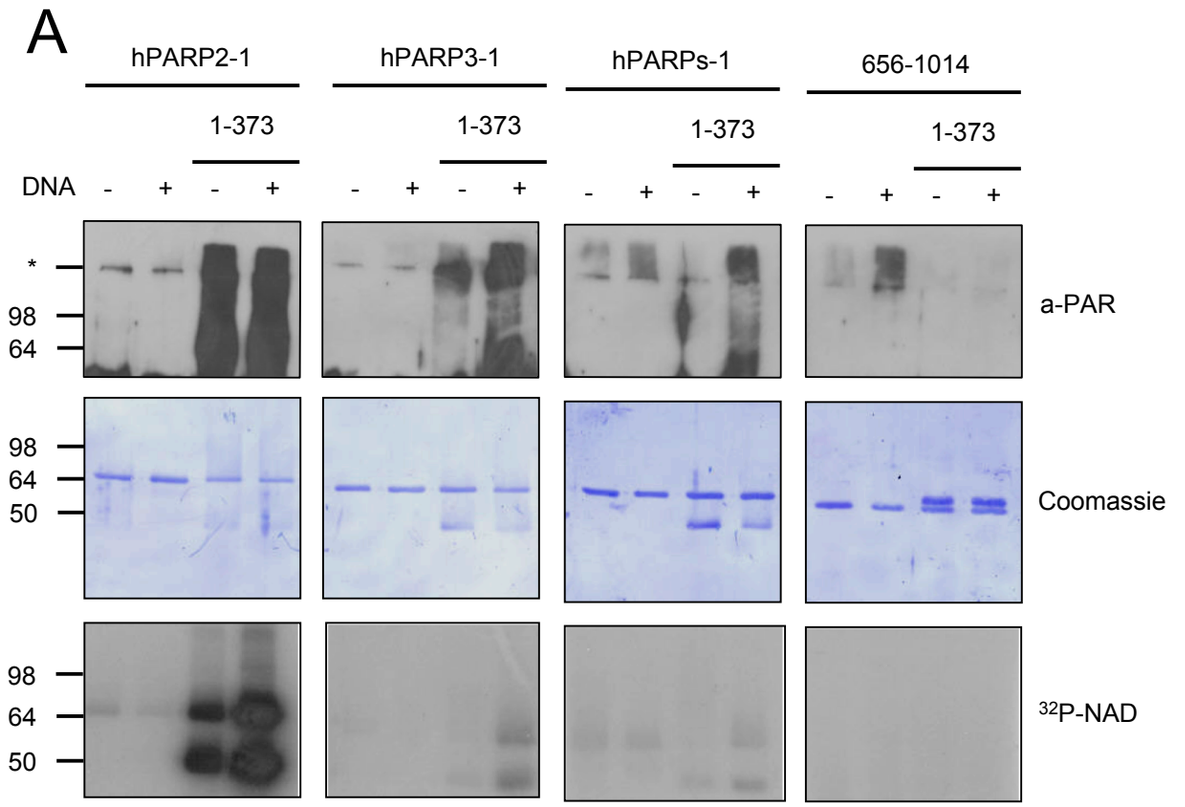
hPARP1
E988K
890/899
DNA
- + - + - +
*
98
a-PAR
*
98
Coomassie
B
1-373
DNA
+ - + + + + + + +
*
98
64
50
98
64
50
98
64
50
32P-NAD
hPARP2-1
1-373
NaCl
NaCl (mM) 0 50 100 150 300 500 1000
hPARP2-1
1-373
1-373 *
+ + +
*
98
64
50
98
64
50
hPARP2-1
1-373
+ + + + +
*
98
64
50
a-PAR
98
64
50
Coomassie
NaCl
NaCl (mM) 0 50 100 150 300
373-1024


A
hPARP2-1
hPARP3-1
hPARPs-1
656-1014
1-373
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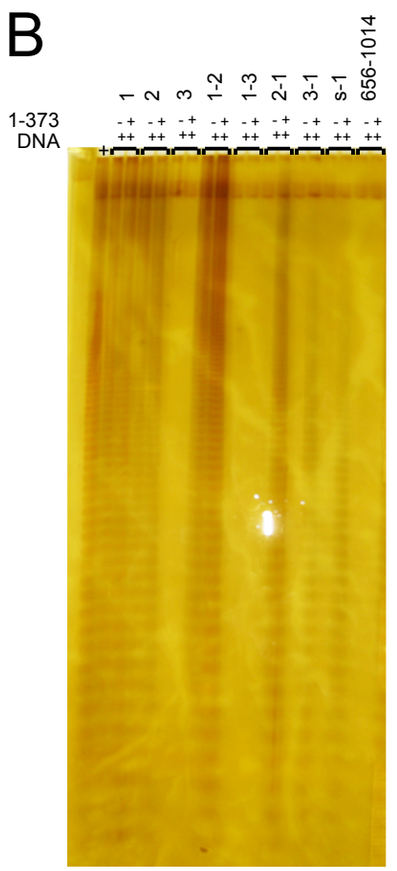
a-PAR
Coomassie
32P-NAD
98
64
50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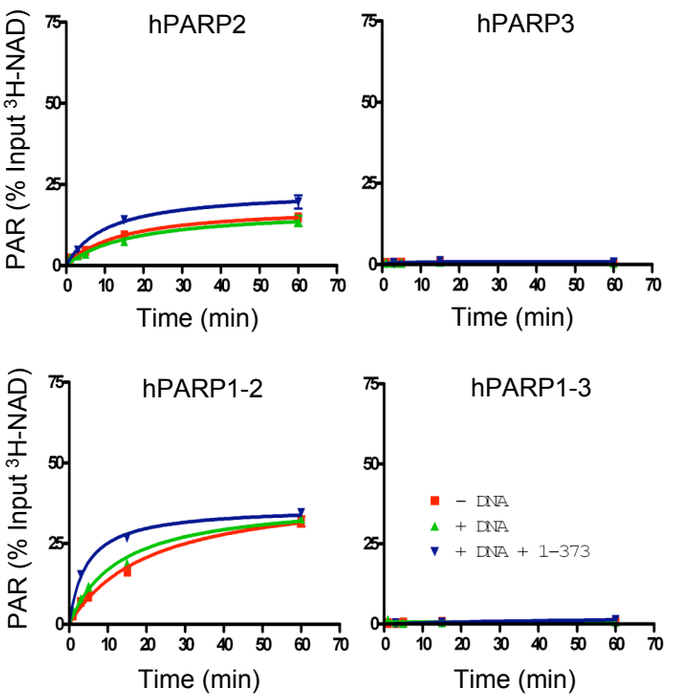
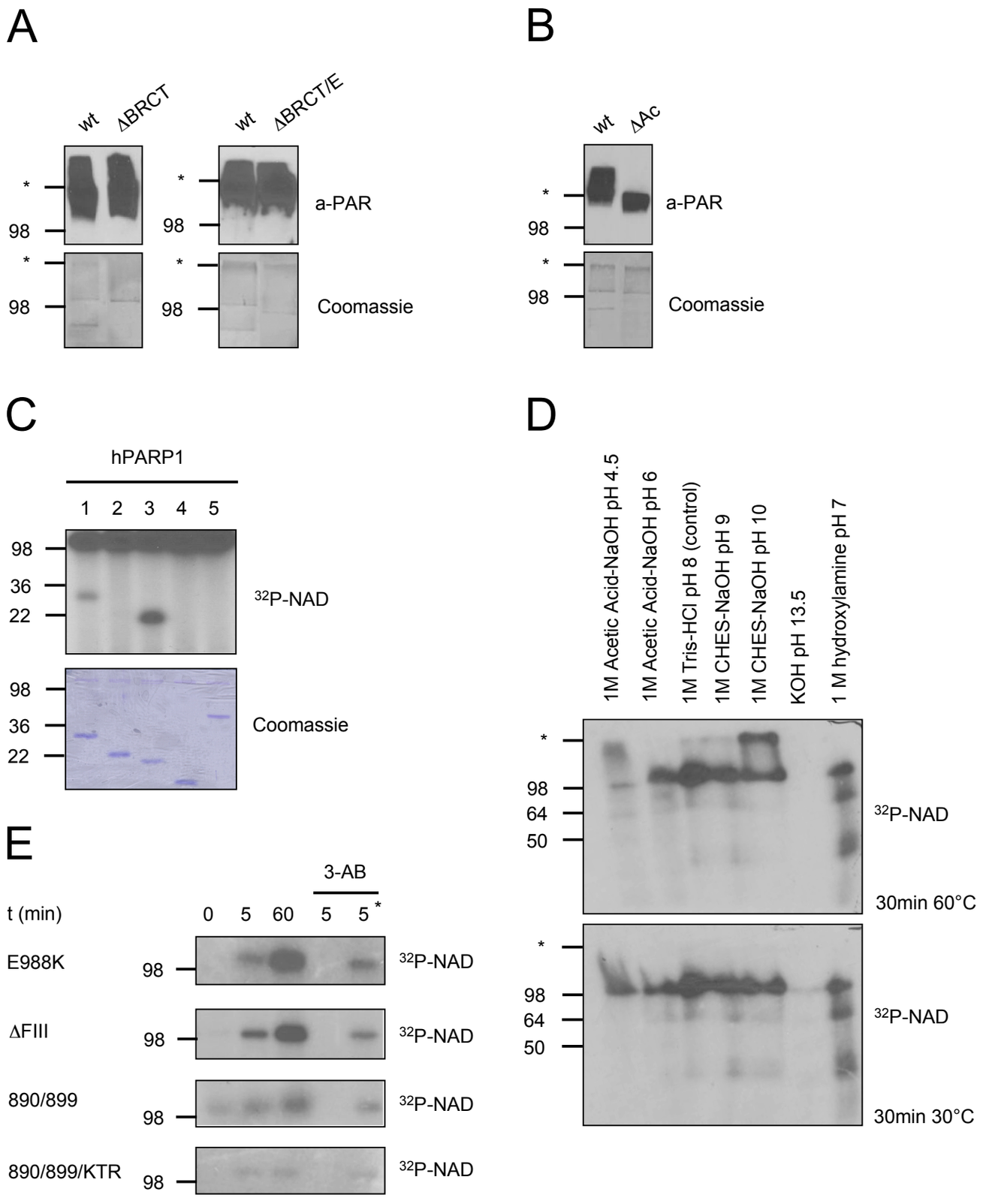
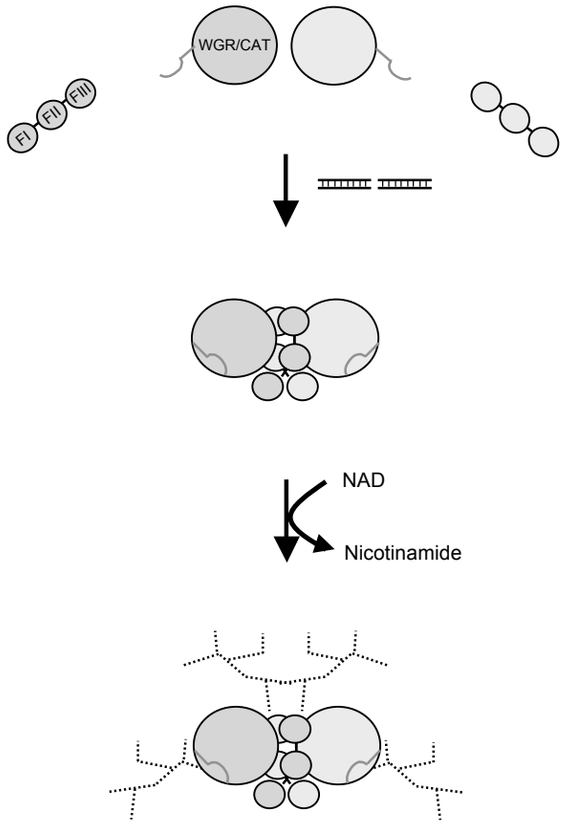
2
3
1-2
1-3
2-1
3-1
s-1
656-1014

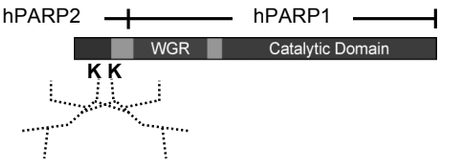

C
hPARP2
hPARP3
hPARP1-2
hPARP1-3
PAR (% Input 3H-NAD)
Time (min)
– DNA
+ DNA
+ DNA + 1–373


A
wt
ΔBRCT
wt
ΔBRCT/E
*
98
a-PAR
Coomassie
B
wt
ΔAc
a-PAR
Coomassie
C
hPARP1
1 2 3 4 5
98
36
22
32P-NAD
Coomassie
D
1M Acetic Acid-NaOH pH 4.5
1M Acetic Acid-NaOH pH 6
1M Tris-HCl pH 8 (control)
1M CHES-NaOH pH 9
1M CHES-NaOH pH 10
KOH pH 13.5
1 M hydroxylamine pH 7
98
64
50
32P-NAD
30min 60°C
30min 30°C
E
3-AB
t (min)
0 5 60 5 5*
E988K
ΔFIII
890/899
890/899/KTR
98
32P-NAD

A


WGR/CAT
FI
FII
FIII
NAD
Nicotinamide

B


hPARP2
hPARP1
WGR
Catalytic Domain
K K

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Identification of lysines 36 and 37 of PARP-2 as targets for acetylation and auto-ADP-ribosylation

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ABSTRACT

Poly-ADP-ribose polymerase-2 (PARP-2) was described to regulate cellular functions comprising DNA surveillance, inflammation and cell differentiation by co-regulating different transcription factors. Using an *in vitro* and *in vivo* approach, we identified PARP-2 as a new substrate for the histone acetyltransferases PCAF and GCN5L. Site directed mutagenesis indicated that lysines 36 and 37, located in the nuclear localization signal of PARP-2, are the main targets for PCAF and GCN5L activity *in vitro*. Interestingly, acetylation of the same two PARP-2 residues reduces the DNA binding and enzymatic activity of PARP-2. Finally, PARP-2 with mutated lysines 36 and 37 showed reduced auto-mono-ADP-ribosylation when compared to wild type PARP-2. Together, our results provide evidence that acetylation of PARP-2 is a key post-translational modification that may regulate DNA binding and consequently also the enzymatic activity of PARP-2.

1. Introduction

Poly-ADP-ribosylation was postulated to function either as reversible covalent post-translational modification of proteins or as non-covalent attachment of free poly-ADP-ribose polymers to proteins (reviewed in Hassa et al., 2006; Hassa and Hottiger, 2008). The enzyme responsible for the synthesis of poly-ADP-ribose was termed poly-ADP-ribose synthetase (PARS) or poly-ADP-ribose polymerase (PARP). PARP-1 has been initially thought to be the only existing enzyme with poly-ADP-ribosylation activity in mammalian cells. However, five additional *parp*-like genes encoding *"bona fide"* PARP enzymes have been identified in recent years (reviewed in Hassa and Hottiger, 2008). These poly-ADP-ribose polymerases (PARP-1 to PARP-6) comprise an ancient family of enzymes, which share a highly conserved catalytic domain (reviewed in Hassa et al., 2006; Hassa and Hottiger, 2008). The active site, also commonly accepted as the 'PARP signature', is formed by an evolutionarily well-conserved sequence of approximately 50 amino acids (residues 859–908 of hPARP-1). The 'PARP signature' contains the NAD acceptor sites and critical residues involved in the initiation, elongation and branching of poly-ADP-ribose polymers (Marsischky et al., 1995; Rolli et al., 1997; Ruf et al., 1998 and reviewed in Hassa et al., 2006; Hassa and Hottiger, 2008). Among the six PARP family members, PARP-2 bears the strongest resemblance of PARP-1 (60% identity in the catalytic domain) (Ame et al., 1999). PARP-2 and PARP-1 can homo- and heterodimerize and display partially redundant functions as indicated by the embryonic lethality of the *parp1-parp2*-double gene disruption in mice (Menissier de Murcia et al., 2003 and reviewed in Schreiber et al., 2006).

Mouse PARP-2 was described as a 66 kDa nuclear chromatin and nuclear matrix associated protein (Ame et al., 1999 and reviewed in Hassa et al., 2006; Hassa and Hottiger, 2008). The amino-terminal part of PARP-

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2 has no significant homology with PARP-1 or with any other PARP family member. These structural differences between PARP-1 and PARP-2 could indicate different substrate specificities of the two proteins (Ame et al., 1999; Johansson, 1999). The amino-terminal region of human and mouse PARP-2 shows high sequence variability and is rich in basic amino acids (27% Lys or Arg). PARP-2 contains an amino-terminal SAP/SAF motif/module (named after scaffold-associated protein/scaffold-associated factor SAF-A/B, Acinus and PIAS, Aravind and Koonin, 2000), a previously undetected eukaryotic module proposed to be involved in sequence- or structure-specific DNA and RNA binding and often associated with different domains involved in the assembly of pre-mRNA processing complexes (Aravind and Koonin, 2000). Similar to PARP-1, PARP-2 was suggested to synthesize poly-ADP-ribose polymers in a DNA-dependent manner (Ame et al., 1999). However, the exact co-enzyme(s) for PARP-2 remains to be elucidated. PARP-2 also displays auto-modification properties similar to PARP-1 and may account for the residual poly-ADP-ribose synthesis observed in *parp-1* knockout cells. PARP-2 has been described in different functions, which are mainly regulated by protein-protein interactions, and it interacts with other proteins mainly through its amino-terminal domain (aa 1–208) (reviewed in Hassa et al., 2006; Hassa and Hottiger, 2008). Similar to PARP-1, PARP-2 is mostly expressed in actively dividing tissues during mouse development, however to a much lower extent (reviewed in Hassa et al., 2006; Hassa and Hottiger, 2008). Unlike PARP-1, the physiological functions of PARP-2 are not yet understood (reviewed in Hassa et al., 2006; Hassa and Hottiger, 2008).

Recently, protein acetylation, mediated by histone acetyltransferases (HATs), such as p300/CBP (CREB-binding protein) and PCAF (p300/CBP-associated factor), has been proposed as a new mechanism for modulating the enzymatic activities of various enzymes, including acetyltransferases, DNA polymerases, DNA nucleases and kinases (Hasan et al., 2002, 2001; Mittal et al., 2006; Sun et al., 2007; Sun et al., 2005). HATs transfer the acetyl group from Acetyl coenzyme A to the epsilon-amino group of a lysine residue on proteins (Yang, 2004b). The steady-state acetylation level of proteins is accomplished by the action of histone acetyltransferases (HATs) and histone deacetylases (HDACs) (Narlikar et al., 2002; Cheung et al., 2000).

PCAF was originally identified as a CBP/p300-binding protein (Yang et al., 1996). PCAF and GCN5L show sequence conservation in the regions responsible for the HAT activity (Candau et al., 1997; Wang et al., 1998). The nuclear histone acetyltransferases PCAF and GCN5L have been shown to acetylate nucleosomal histones *in vitro* and *in vivo*. PCAF as well as GCN5L exist as components of large multisubunit HAT/chromatin remodeling complexes (Ogryzko et al., 1996; Forsberg et al., 1997). These complexes possess global histone acetylation activity and locus-specific co-activator functions together with FAT activity on non-histone substrates. There are large numbers of known substrates for PCAF and GCN5L including many non-histone proteins, however, histones are considered to be their major targets (for a review see Yang, 2004a). Thus, the biological functions of PCAF and GCN5L cover a wide range of tasks and render them indispensable for the normal function of cells. In vertebrates, it became clear that the two proteins are not expressed in every cell type and even if they are coexpressed in the same cell, their relative expression level can vary substantially.

We have recently reported that PARP-1 can be specifically acetylated by p300/CBP. Here, we provide evidence that PARP-2 is acetylated in mammalian cells and *in vitro* and that the two lysine residues K36 and K37 are the major direct target sites for PCAF and GCN5L-mediated acetylation. Interestingly, acetylation of PARP-2 strongly reduces the DNA binding and enzymatic activity of PARP-2. Finally, PARP-2 with mutated lysines 36 and 37 showed reduced auto-mono-ADP-ribosylation when compared to wild type PARP-2, indicating that lysines 36 and 37 may also serve as target sites for auto-ADP-ribosylation of PARP-2.

2. Materials and methods

2.1. Plasmids

Mammalian expression vectors for wild type mPARP-2 and different mPARP-2 mutants (K19/20R, K36/37R, K19/20/36/37R, K36R and K37R) were obtained by cloning the corresponding PCR products into pphCMV-HA. Baculovirus expression vectors for His-tagged mPCAF and hGCN5L were obtained by cloning the corresponding PCR products into pBACPAK8 (Clontech Laboratories). Baculovirus expression vectors for His-tagged wild type PARP-2 and the different PARP-2 mutants were obtained by cloning the corresponding PCR products into pphBACPAK9-MC-PrSc-HA-HIS or pphBACPAK9-MC-PrSc-HIS. The corresponding baculoviruses were generated using the BACPAK6-based bacmid system from Clontech Laboratories. All PARP-2 mutants were generated by a site directed mutagenesis procedure and confirmed by sequencing. Mammalian expression vectors for mPCAF and hGCN5L were obtained by cloning the corresponding PCR products into pphCMVkm (Hassa et al., 2005). The bacterial expression vectors for GST-HAT full-length fusion proteins (mPCAF and hGCN5L) were obtained by cloning the corresponding PCR products into pGex6P1.

2.2. Expression and purification of recombinant proteins

Recombinant hp300, mCBP, mPCAF and hGCN5L, as well as mPARP-2 full-length, fragments and mutant proteins were expressed as carboxy-terminal His-tagged proteins in *Sf21* insect cells as described previously (Hassa et al., 2003, 2005). Recombinant proteins were purified by standard nickel-NTA affinity chromatography according to the manufacturer's protocol in the presence of 1 M NaCl/0.5% NP-40. GST-tagged proteins were expressed in *E. coli* strain BL21-DE3-Gold as described previously (Hassa et al., 2003, 2005). Recombinant histones H2A/H2B (refolded) were a kind gift from Tobias Stuwe (European Molecular Biology Laboratory (EMBL), Gene Expression Unit, 69117 Heidelberg, Germany). All purified proteins were analyzed by Coomassie staining and confirmed by Western blot analysis using the corresponding antibodies.

2.3. Cell culture and transient transfections

HEK293T cells were grown in Hepes-buffered DMEM-Glutamax-I (Invitrogen) containing 4.5 g/L glucose and 10% FCS US/certified (Invitrogen) and supplemented with 50 U/ml penicillin, 50 μg/ml streptomycin (Invitrogen) and MEM-non-essential amino acids (MEM NEAA, Invitrogen). Cells were transfected using calcium phosphate procedures as previously described (Perkins et al., 1997).

2.4. Immunoprecipitation and Western blot analysis

Co-immunoprecipitation for PARP-2 and mPCAF or hGCN5L was performed as described previously (Hassa et al., 2003, 2005). In brief, IP-buffers contained 50 mM Tris–HCl (pH 8.0), 120 mM NaCl, 0.5% NP-40, 0.5 mM DTT, protease inhibitors and, where indicated, histone deacetylase inhibitors (2 μM TSA, 2 mM sodium butyrate, 5 mM nicotinamide). Western blot analyses were performed as described previously (Hassa et al., 2003, 2005). Anti-myc-9E10 (sc-2027) antibodies were obtained from Santa Cruz Biotechnology, anti-HA (MMS-101P) was obtained from COVANCE SA, anti-acetyl-lysine antibodies (AKL5C1) were provided by Dr. Ito (RIKEN, Japan) or purchased from Santa Cruz Biotechnology (sc-32268) (dilution for WB 1:5000). Antibodies against mouse PARP-1 and PARP-2 were generated in house (the generation of antibodies against mouse and human PARP-1 was described previously Hassa et al., 2005; Petrilli et al., 2004).

2.5. In vitro interaction and GST pull-down assays

Purified recombinant proteins fused to GST were bound to Glutathione Sepharose 4B beads according to the manufacturer's protocols (Amersham Biosciences). GST pull-down assays were performed as described (Hassa et al., 2003, 2005). GST pull-down-buffers contained: 50 mM Tris–HCl (pH 8.0), 150 mM NaCl, 0.5% NP-40, 0.5 mM DTT and protease inhibitors. Bound proteins were dissolved by boiling and subsequently analyzed by Western blot.

2.6. Histone-acetyltransferase (HAT) assays

2.6.1. HAT assays used for identification of acetylated lysines and for auto-mono-ADP-ribosylation assays

One microgram of baculo-purified PARP-2 full-length or 2 μg of baculo-purified PARP-2 fragments were incubated with 0.5 μg of baculo-purified histone acetyltransferases and 1.5 nmol radiolabelled (0.1 μCi/mmol [^{14}C], MC 269, Movarek Biochemicals) or unlabelled Acetyl-CoA in 30 μl HAT-Buffer (50 mM Tris–HCl (pH 8.0), 5% (v/v) glycerol, 50 mM NaCl, 0.1% (v/v) NP-40, 1 mM DTT, 1 mM PMSF, 2 mM sodium butyrate) at 30 °C for 30 min. Proteins were subsequently separated by SDS-PAGE. Gels were stained with Coomassie and subjected to autoradiography, or Western blot analysis was performed with the indicated antibodies.

2.6.2. HAT assay used for electro-mobility shift assays (EMSA) and poly-ADP-ribose product analysis

Five microgram of purified GST, GST-mPCAF or GST-hGCN5L and 10 μg of target protein (PARP-2 full-length, PARP-2 aa 1–91 or aa 1–209) were incubated in HAT assay buffer containing 50 mM Tris–HCl (pH 7.8), 100 mM NaCl, 10% (v/v) glycerol, 1 mM DTT, 1 mM PMSF, 0.1 mM EDTA (pH 8.0), 2 mM sodium butyrate and 50 μM Trichostatin A at 30 °C for 2 h, in the presence or absence of 100 μM Acetyl-CoA. The final reaction volume was 50 μl. GST, GST-mPCAF or GST-hGCN5L were then bound to Glutathion Sepharose 4B beads and the supernatants were used for poly-ADP-ribosylation and electro-mobility shift assays. As control, aliquots of the reaction mixtures were subsequently separated on 12% SDS-PAGEs and processed for Coomassie staining and Western blot analysis.

2.6.3. Tris-glycine based electro-mobility shift assays (EMSA) procedure

Acetylated or non-acetylated (i.e. reaction was performed without Acetyl-CoA) wild type full-length PARP-2 or DBD fragments of PARP-2 were incubated with flap DNA oligonucleotide in 20–25 μl EMSA binding buffer at 25 °C for 10–20 min. Molar ratios of oligonucleotide to protein are indicated in the figure legends. EMSA-binding buffer: 30 mM Hepes (pH 7.9), 100 mM NaCl, 10 mM $MgCl_2$ and 5% sucrose. To equilibrate pH, NaCl, Acetyl-CoA and protein concentrations, the reaction was compensated with HAT Buffer (+/− Acetyl-CoA) containing BSA. Reactions were stopped with 10X sucrose based gel loading buffer. Complexes were subsequently resolved on native 6-8% polyacrylamide gels (AA:BA, 19:1 or 40:1, "UltraPure-RNA-Grade" Acrylamide, Ambion/Amersham Bioscenciences) in Tris-glycine buffer (250 mM glycine, 50 mM Tris–HCl, (pH 8.3)) at 4 °C for 5–8 h at 4 W. Free DNA probes and DNA/PARP-2 complexes were stained with toluidine blue O (TBO).

2.6.4. In vitro poly-ADP-ribosylation assays

If not otherwise stated, for poly-ADP-ribosylation assays 1 μg of purified PARP-2 was incubated with 400 μM NAD^+, 2 μCi ^{32}P-labelled NAD^+ (Amersham Biosciences) and 1 μg activated DNA (obtained from Trevigen) or flap-oligonucleotides at 30 °C for 10 min (Buffer: 50 mM Tris–HCl (pH 8.0), 4 mM $MgCl_2$, 0.5 mM DTT and protease inhibitors) in a total volume of 50 μl. Proteins were resolved by SDS-PAGE, stained with Coomassie and exposed to X-ray films (Contatyp).

2.6.5. Poly-ADP-ribose polymer product analysis

Hundred nanogram of *in vitro* acetylated or non-acetylated PARP-2 full-length proteins were pre-incubated in 25 μl poly-ADP-ribosylation reaction buffer in the presence of increasing amounts of DNA flap-oligonucleotide (as indicated in the figure legends) for 20 min at room temperature. 75 μl of poly-ADP-ribosylation reaction buffer containing 166.6 mM NAD^+ was then added to the reaction (final NAD^+ concentration: 125 mM) and the mixture further incubated in a thermo mixer for 5 min at 30 °C and 550 rpm. Reaction buffer: 100 mM Tris–HCl (pH 7.8), 10 mM $MgCl_2$ 125 mM NaCl, 2 mM DTT, +/− purified

recombinant histone H2A/H2B protein. The molar ratio of PARP-2 to histone H2A/H2B protein was 1:2. The reactions were stopped by addition of 1 volume 20% ice-cold TCA. Poly-ADP-ribose polymers were subsequently analyzed on 20% UREA-PAGE (AA:BA, 40:1, "UltraPure-RNA-Grade" Acrylamide, Ambion/Amersham Bioscenciences). Silver staining of poly-ADP-ribosylation products was performed according to manufacturer's procedures (PIERCE; Color Silver Stain Kit).

2.6.6. Oligonucleotides used in this study

Flap-dsDNA 106mer:

- 5′-AAGGGCAAGGCTGCTGCTGTGGAGTGTTGCATTCC ATATATAATATATAAAA-3′,
- 5′-TACCTGCTGGACCCTGCTGTGGGCTGGAGAACAAGG TGATCTGCGCTCTGGTCGT-3′,
- 5′-ACGACCAGAGCGCAGATCACCTTGTTCTCCAGCCCA CAGCAGGGTCCAGCAGGTACCTGGCCGCCCAGAGAGGA ATGCAACACTCCACAGCAGCAGCCTTGCCCTT-3′.

Upon hybridization, the Flap-dsDNA 106mer was resolved either on 3% agarose gels or 10% native-PAGE, the Flap-dsDNA product was cut out, eluted and purified using a desalting column.

3. Results

3.1. PARP-2 is acetylated in vivo

Acetyltransferases are known to modify a variety of proteins, such as histones and transcription factors (Sterner and Berger, 2000). To investigate whether PARP-2 might be acetylated *in vivo*, we immunoprecipitated overexpressed HA-tagged PARP-2 under high salt conditions from whole cell extracts of 293T cells. Western blot analysis using a specific anti-AcK antibody revealed that PARP-2 was acetylated *in vivo* (Fig. 1A and Supplementary Fig. 1). No increase in acetylation was observed in the presence of the HDAC inhibitors sodium butyrate, nicotinamide or trichostatin A (Supplementary Fig. 2), suggesting that acetylation of

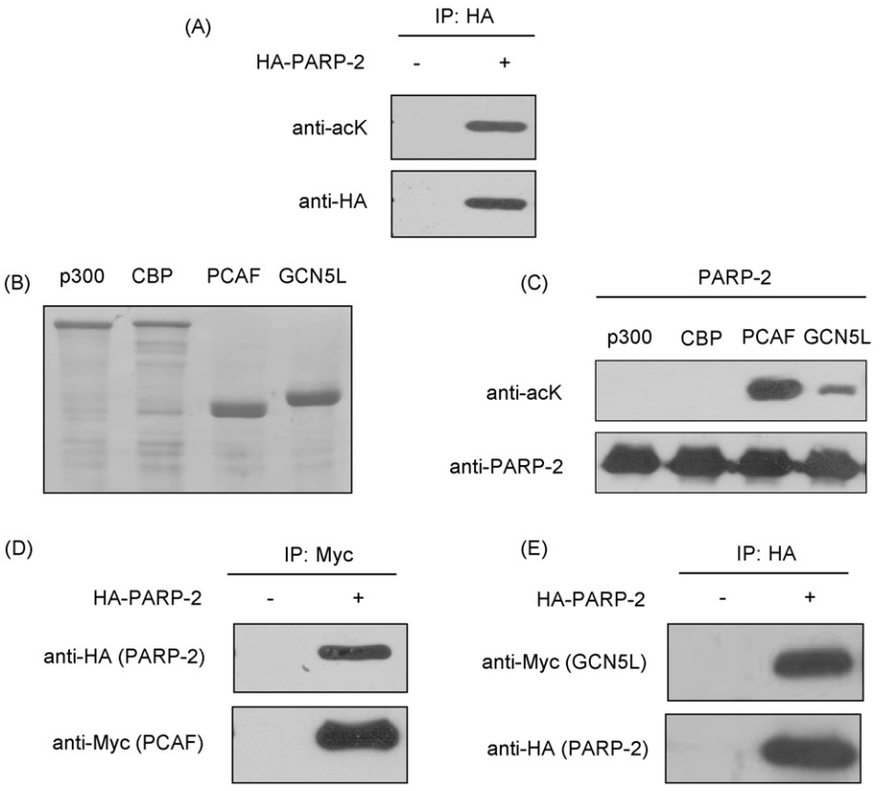

Fig. 1. PARP-2 is acetylated *in vivo* and *in vitro* by PCAF and GCN5L. (A) HEK293T cells were transfected with HA-tagged PARP-2 as indicated, then immunoprecipitation was performed using a monoclonal anti-HA antibody. Samples were separated by SDS-PAGE and transferred to a PVDF-membrane, which was then incubated with an antibody specific to acetylated lysine residues. Untransfected cells were used as negative control for immunoprecipitation. (B) Recombinant His-tagged histone acetyltransferases (HATs) were expressed in *Sf21* cells and purified with NTA-Nickel beads. Purified proteins were resolved by SDS-PAGE and stained with Coomassie. (C) Purified PARP-2 was subjected to *in vitro* acetylation by p300, CBP, PCAF or GCN5L. Proteins were subsequently separated by SDS-PAGE and transferred to a PVDF membrane, followed by incubation with the indicated antibodies. Under these conditions, PARP-2 is acetylated mainly by PCAF and to a lower extent by GCN5L. (D) PARP-2 interacts with PCAF *in vivo*. Myc-tagged PCAF was co-expressed with HA-tagged PARP-2 in HEK293T cells and immunoprecipitated using an anti-Myc antibody. Bound PARP-2 was detected using an anti-HA antibody. Untransfected cells served as IP control. (E) PARP-2 interacts with GCN5L *in vivo*. Myc-tagged GCN5L was co-expressed with HA-tagged PARP-2 in HEK293T cells. PARP-2 was immunoprecipitated using an anti-HA antibody and bound GCN5L was detected by Western blot against the Myc-tag.

PARP-2 is a stable modification in mammalian cells. Several attempts to immunoprecipitate endogenous PARP-2 failed with all available anti-PARP-2 antibodies due to very low - expression levels of endogenous PARP-2 in the different cells tested, including HEK293T, HeLa, NTERA-2, NCCIT, GC-1/2, Jurkat or THP-1 cells, despite the fact that PARP-2 mRNA was detected in these cells by RT-PCR. These observations indicate that the translation or degradation of PARP-2 is tightly controlled (suppl Fig. 3 and data not shown).

3.2. PARP-2 is acetylated in vitro by both PCAF and GCN5L and forms a complex with PCAF and GCN5L in vivo

Next, we tested whether PARP-2 can be acetylated *in vitro*. Recombinant purified full-length PARP-2 was incubated with different recombinant purified acetyltransferases (Fig. 1B) in the presence of Acetyl-CoA and resolved by SDS-PAGE followed by Western blot analysis using the indicated antibodies. PARP-2 was acetylated *in vitro* by PCAF and to a weaker extent by GCN5L, but not by p300, CBP, HAT-1 or TIP-60 (Fig. 1C and data not shown). An estimation of the acetylation efficiency revealed that only a minor portion of PARP-2 was acetylated under the tested conditions *in vitro*. PCAF and GCN5L are known to have weak acetyltransferase activity compared to p300 and CBP.

Since these results strongly suggested that the two histone acetyltransferases PCAF and GCN5L might physically interact with PARP-2 *in vivo*, we co-expressed Myc-tagged PCAF and HA-tagged PARP-2 in HEK293T cells, immunoprecipitated PCAF complexes from whole cell extracts and tested the presence of HA-tagged PARP-2 by Western blot analysis using an anti-HA antibody. PARP-2 formed, indeed, a complex with PCAF *in vivo* (Fig. 1D). Similar experiments with GCN5L and PARP-2 revealed that also GCN5L associates with PARP-2 (Fig. 1E).

3.3. PARP-2 is acetylated at K36 and K37.

In order to map the PARP-2 domains, which are acetylated by PCAF, *in vitro* acetylation assays were performed with different PARP-2 fragments corresponding to amino acids 1–91, aa 92–207, aa 1–207 or aa 208–559 in the presence of radiolabelled Acetyl-CoA. These experiments revealed that PCAF strongly acetylated the PARP-2 domain corresponding to aa 1–91 (Fig. 2A). To identify the target lysines acetylated by PCAF, all potential PCAF/GCN5L-KK-type acetylation motifs within the DNA binding domain of PARP-2 were mutated by site directed mutagenesis (Fig. 2B). We expressed the corresponding mutant versions of PARP-2, K19/20R, K36/37R and K19/20/36/37R, in *Sf21* insect cells. PARP-2 wild type and mutant proteins were subsequently purified and acetylated *in vitro* by PCAF and GCN5L in the presence of radiolabelled Acetyl-CoA. Acetylated proteins were resolved by SDS-PAGE and subjected to autoradiography (Fig. 2 C and data not shown). These results revealed that lysine K36 and/or K37 are indeed strong candidates for acetylation by PCAF and GCN5L. Detection of actylated lysines by Western blot analysis using an anti-AcK antibody revealed a weak but detectable signal for the K36/37R mutant after long exposure, possibly representing an additional, not yet identified acetylation site of PARP-2 (Supplementary Fig 4). To further confirm that both lysines are targets for acetylation, we repeated similar assays with single mutants of PARP-2 (K36R and K37R, respectively). These experiments revealed that indeed both lysines are acetylated *in vitro* by PCAF and GCN5L (Fig. 2D).

We furthermore investigated whether lysines 36 and 37 of PARP-2 may serve as the main acceptor sites for acetylation *in vivo*. Overexpressed HA-tagged PARP-2 wild type or mutants were immunoprecipitated under high salt conditions from whole cell extracts of 293T cells in the presence of deactylase inhibitors (Fig. 2E). The presence of acetylated forms of PARP-2 was tested by Western blot analysis using an anti-AcK antibody. While wild type PARP-2 and the PARP-2 mutant K19/20R were acetylated *in vivo*, the PARP-2 mutants harboring the two lysines K36 and K37 mutated to arginines were no longer acetylated (Fig. 2D). Overexpression of PCAF or GCN5L did not increase acetylation of PARP-2, indicating that the endogenous levels of PCAF or GCN5L were high enough to maintain PARP-2 in the acetylated status (data not shown). During the course of our experiments we observed that the K36/37R PARP-2 mutant is, in contrast to its wild type counterpart, not localized to the nucleus (Supplementary Fig. 5 and manuscript in submission). Since both PCAF and GCN5L were mainly localized in the nucleus under the tested conditions (Supplementary Fig. 5), the absence of acetylation of the PARP-2 mutants K36/37R and K19/20/36/37R was very likely due to their localization in a different subcellular compartment. The additional involvement of the two mentioned lysines in the nuclear translocation of PARP-2 thus did not allow further *in vivo* analysis of the indicated mutants.

3.4. Acetylation of Lysines 36 and 37 inhibits DNA binding of PARP-2

It was hypothesized earlier that the amino-terminal part of PARP-2 might contain a DNA binding domain (DBD). Thus, the amino-terminal fragments of PARP-2 containing the postulated DBD alone (aa 1–91) or together with the WGR domain (aa 1–207) were expressed in *Sf21* insect cells, purified, and tested in electro-mobility shift assays (EMSA) for their binding to a flap DNA oligonucleotide. Previous reports and own experiments had revealed that PARP-2 preferentially binds to DNA oligonucleotides containing long flaps or gaps but only very weakly to nicked, short gapped and single stranded DNA (Ame et al., 1999; Dantzer et al., 2006b and data not shown). The EMSAs performed confirmed that PARP-2 is able to bind DNA and that the aa 1–91 fragment is sufficient for this function (Fig. 3 A and B). The WGR domain alone was not able to bind to DNA. *In vitro* acetylation of full-length PARP-2 or its DBD by PCAF or GCN5L and subsequent repetition of the experiments with acetylated proteins revealed that the binding to DNA was severely reduced by acetylation (Fig. 3 B and C). Acetylation of the different tested proteins was controlled by Western blot analysis using an anti-AcK antibody (Supplementary Fig. 6).

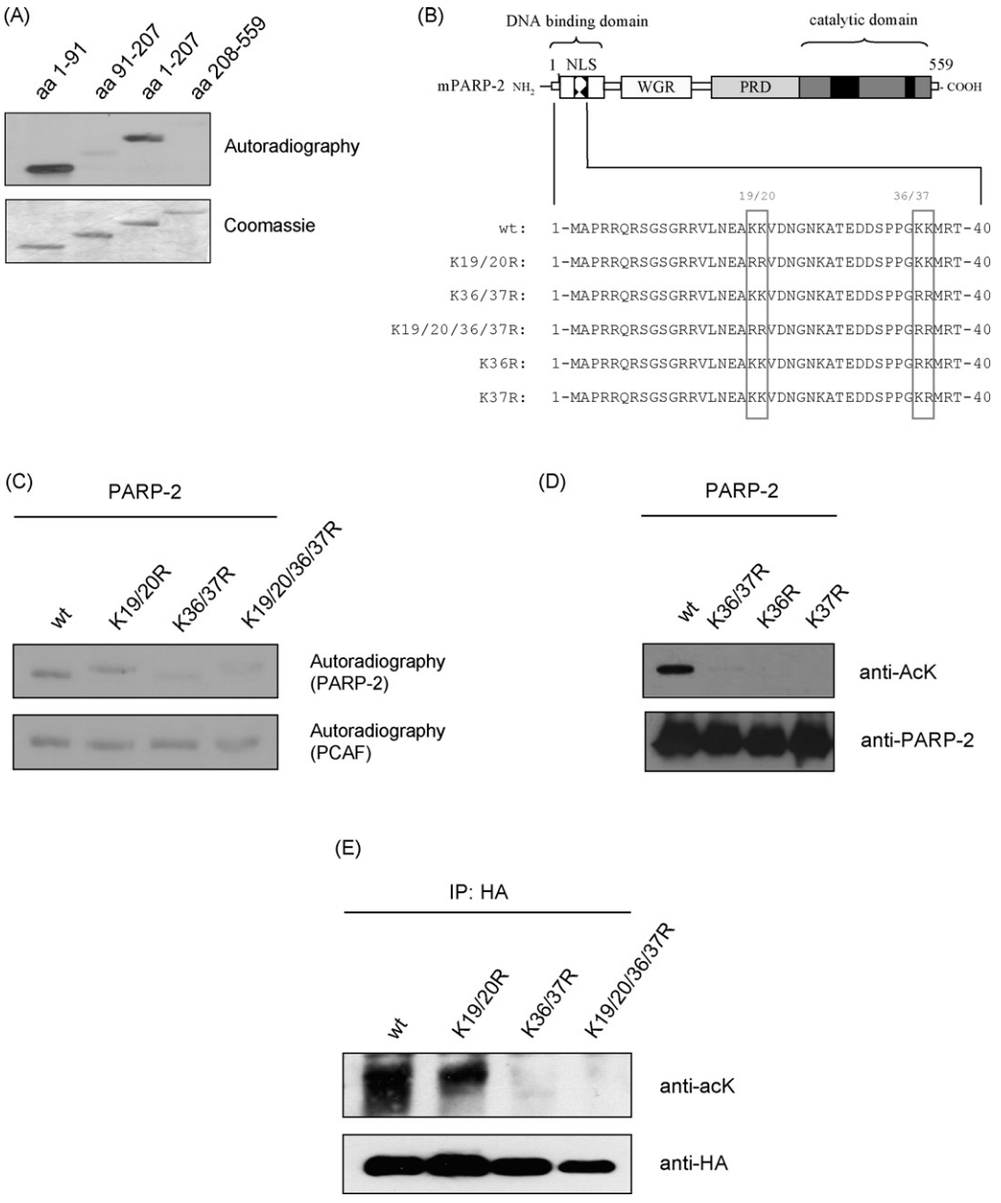

Fig. 2. PARP-2 is acetylated at lysines 36 and 37. (A) The postulated DNA binding domain of PARP-2 (aa 1–91) is acetylated by PCAF *in vitro*. Purified recombinant PARP-2 fragments aa 1–91, aa 92–207, aa 1–207 and aa 208–559 expressed in *Sf21* cells were *in vitro* acetylated with PCAF and analyzed by autoradiography. (B) Schematic illustration of PARP-2 mutant proteins used in this study. Lysines K19, K20, K36 and K37 were changed to arginine using site directed mutagenesis. Single, double and quadruple mutants were generated. (C) Purified recombinant wild type (wt) PARP-2 or the indicated double and quadruple mutants were incubated with purified recombinant PCAF in the presence of radiolabelled Acetyl-CoA. Auto-acetylated PCAF and acetylated PARP-2 were detected by autoradiography. (D) Purified recombinant wild type (wt) PARP-2 or the indicated single and double mutants were incubated with purified recombinant PCAF in the presence of Acetyl-CoA. Acetylated PARP-2 was analyzed by Western blot using an anti-AcK antibody. (E) The PARP-2 mutants K36/37R and K19/29/36/37R are no longer acetylated *in vivo*. HA-tagged wild type (wt) PARP-2 and the mutant proteins K19/20R, K36/37R and K19/20/36/37R were transfected into HEK293T cells, then immunoprecipitation was performed using an anti-HA antibody. Proteins were separated by SDS-PAGE and subsequently tested for acetylation by Western blot analysis with an anti-AcK specific antibody.

3.5. Acetylation of PARP-2 inhibits its enzymatic activity

In order to test whether acetylation also inhibits the enzymatic activity of PARP-2, recombinant full-length wild type PARP-2 was acetylated *in vitro* with purified GST-PCAF and GST-hGCN5L and subsequently tested in poly-ADP-ribosylation assays in the presence of 125 μM NAD^+. Analysis of the poly-ADP-ribose polymer products revealed that the activity of PARP-2 was strongly stimulated by long gap and flap containing DNA oligonucleotides (Fig. 4A and B, left panels and data not shown), although PARP-2 was able to auto-ADP-ribosylate itself and to form poly-ADP-

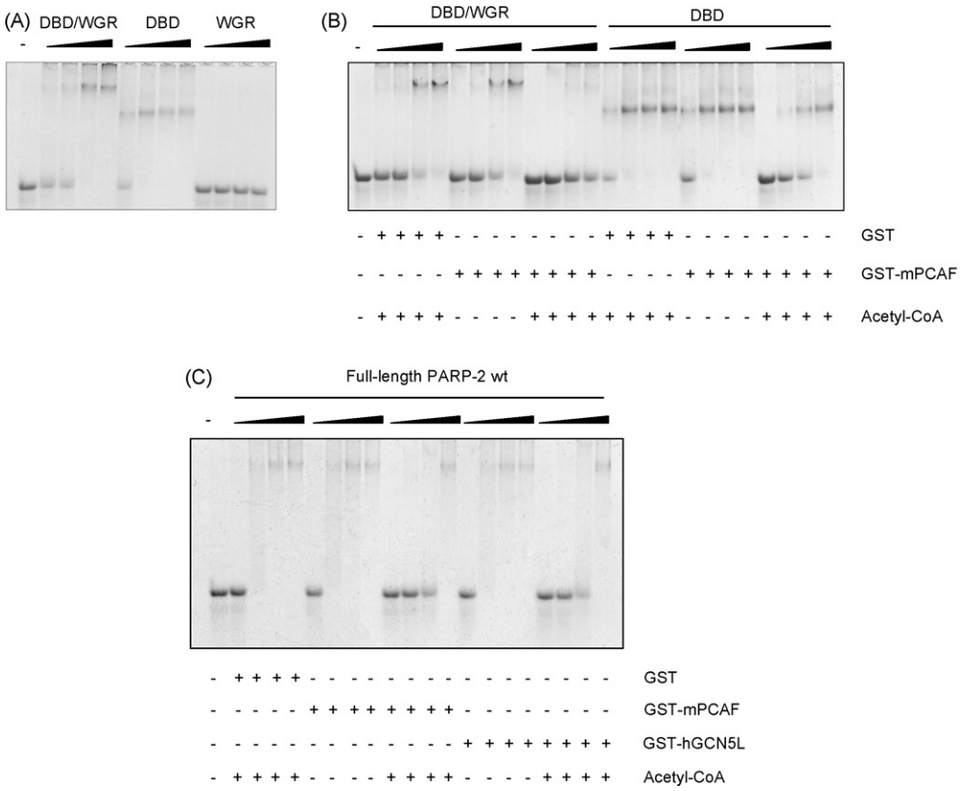

Fig. 3. Acetylation of PARP-2 by mPCAF and hGCN5L inhibits PARP-2 DNA binding activity. (A) Increasing amount of purified fragments representing the postulated DNA binding region of PARP-2 were tested in electro-mobility shift assays (EMSA) for binding to a flap DNA oligonucleotide. Molar ratios of oligonucleotide to PARP-2 fragment: 1:1, 1:2, 1:4 and 1:8. (B) Increasing amount of purified fragments representing the DNA binding region of PARP-2 were acetylated *in vitro* with purified GST-mPCAF and subsequently tested in EMSA for binding to a flap DNA oligonucleotide. Molar ratios of oligonucleotide to PARP-2 fragment: 2:1 1:1, 1:2 and 1:4. (C) Increasing amount of purified full-length wild type PARP-2 was acetylated *in vitro* with purified GST-mPCAF or GST-hGCN5L and subsequently tested in EMSA for binding to a flap DNA oligonucleotide. Molar ratios of oligonucleotide to PARP-2 fragment: 1:1, 1:2, 1:3 and 1:4.

ribose polymers in the complete absence of DNA (see also Fig. 5). Acetylation of PARP-2 by both PCAF and GCN5L severely inhibited the enzymatic activity of PARP-2 (Fig. 4 A and B, left panels).

Histone H2A and H2B were recently suggested to serve as preferred substrates for PARP-2 (Ame et al., 1999; Dantzer et al., 2006a). Addition of H2A/H2B to poly-ADP-ribosylation reactions stimulated polymer formation of shorter poly-ADP-ribose polymers by PARP-2, which was again severely inhibited by acetylation of PARP-2 (Fig. 4A and B, right panels).

3.5.1. Lysines 36 and 37 of PARP-2 are important for auto-mono-ADP-ribosylation

To investigate the relevance of lysines 36 and 37 for auto-mono-ADP-ribosylation, wild type and PARP-2 mutant K36/37R were expressed in *Sf21* insect cells and purified in the presence of ethidium bromide to avoid copurification of DNA. Equal amounts of wild type and PARP-2 mutant K36/37R were subsequently used in *in vitro* auto-mono-ADP-ribosylation assays in the presence of 2 μM - NAD^+ to favor auto-mono-ADP-ribosylation of PARP-2 (Fig. 5). Remarkably, auto-ADP-ribosylation of the mutant K36/37R was substantially reduced, but not completely impaired. As observed before, both wild type and mutant PARP-2 showed a basal enzymatic activity under these conditions, which was not dependent on DNA or RNA (data not shown). Time course experiments confirmed the initial observation that mutant K36/37R was, even after increasing the incubation time, not able to mono-ADP-ribosylate itself to the same extent as wild type PARP-2 (Fig. 5). Together these experiments revealed that lysines 36 and 37 are important for mono-ADP-ribosylation of PARP-2, potentially by serving as acceptor sites for ADP-ribose. Interestingly, the same experiments in the presence of 100 μM NAD^+ revealed that the PARP-2 mutant K36/37R was still able to synthesize poly-ADP-ribose polymers (Fig. 5).

4. Discussion

The aim of this study was to investigate whether PARP-2 might be regulated by post-translational modifications such as acetylation. Here, we provide both biochemical and functional evidence for acetylation of PARP-2 by PCAF and GCN5L. We identified two lysine residues (K36 and K37)

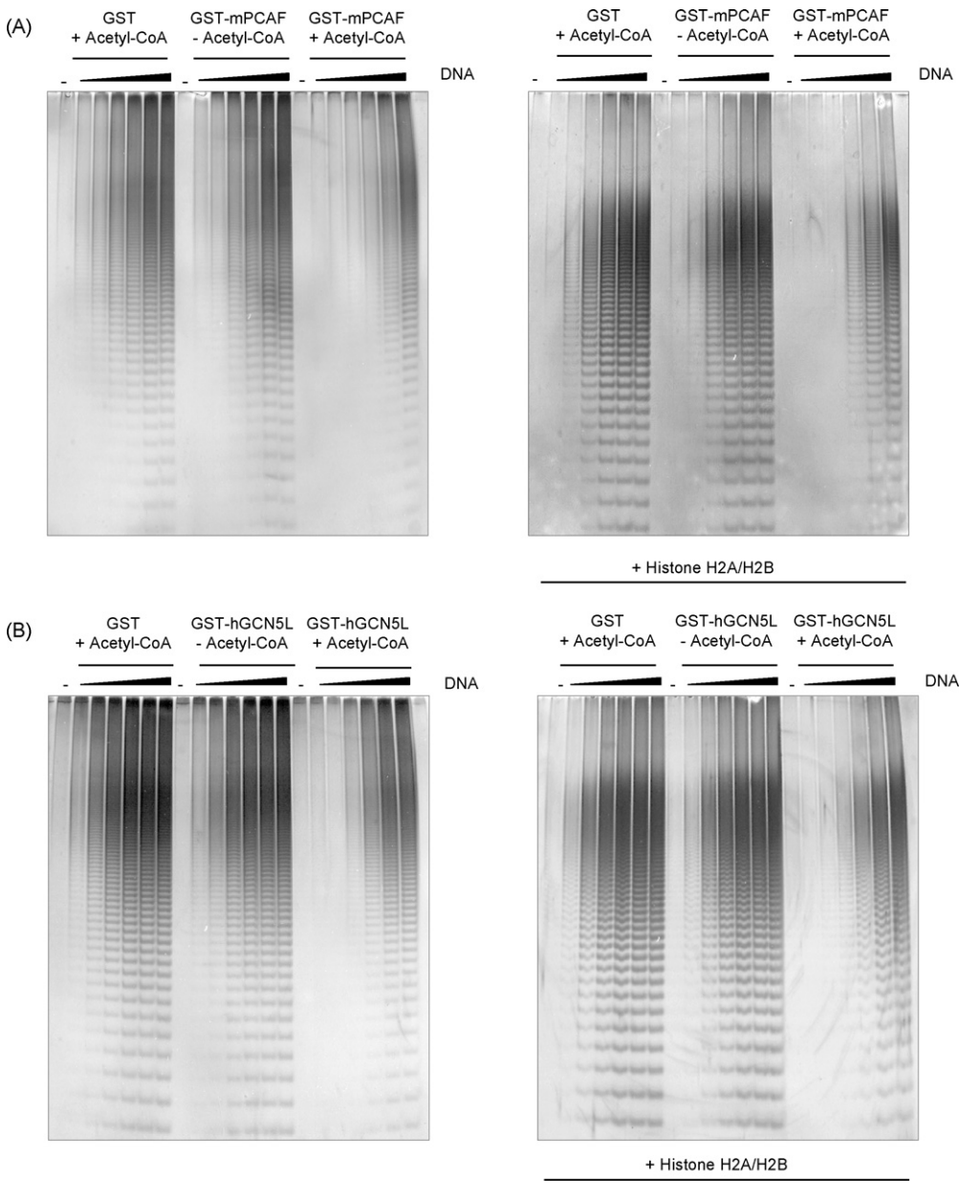

Fig. 4. Acetylation of PARP-2 by mPCAF and hGCN5L inhibits PARP-2 enzymatic activity. Product analysis of poly-ADP-ribose produced *in vitro* by acetylated and non-acetylated PARP-2 in the absence (left panels) or presence (right panels) of histones H2A/H2B. (A) Full-length wild type PARP-2 was acetylated *in vitro* with purified GST-mPCAF and subsequently tested in poly-ADP-ribosylation assays. Poly-ADP-ribose products were purified, separated by 20% Urea-PAGE and processed for silver staining. Molar ratios of PARP-2 to flap-DNA oligonucleotide: 2:1, 1:1, 1:2, 1:3, 1:4 and 1:8. (B) Full-length wild type PARP-2 was acetylated *in vitro* with purified GST-hGCN5L and poly-ADP-ribosylation was tested as described for Fig. 4A.

that are acetylated in mammalian cells and are also direct substrates for PCAF *in vitro*. *In vitro* acetylation of the DNA binding domain (aa 1–91) of PARP-2 inhibited its binding to DNA oligonucleotides. Moreover, *in vitro* acetylation of full-length PARP-2 reduced its enzymatic activity. Remarkably, mutation of K36 and K37 substantially reduced the auto-mono-ADP-ribosylation of PARP-2 at low NAD^+ concentrations *in vitro*, suggesting that these two residues might serve as acceptor sites for ADP-ribosylation.

Acetylation of lysine residues was described to be a reversible process providing dynamic responses to extra- and intracellular signaling events (Yang and Seto, 2007). The proteins catalyzing the deacetylation reaction have been termed histone deacetylases (HDAC) (Gregoretti et al., 2004; Blander and Guarente, 2004). Interestingly, overexpression of HDAC class 1 to 4 did not reduce the observed acetylation of PARP-2 *in vivo* (data not shown) nor did the addition of HDAC inhibitors increase the acetylation levels

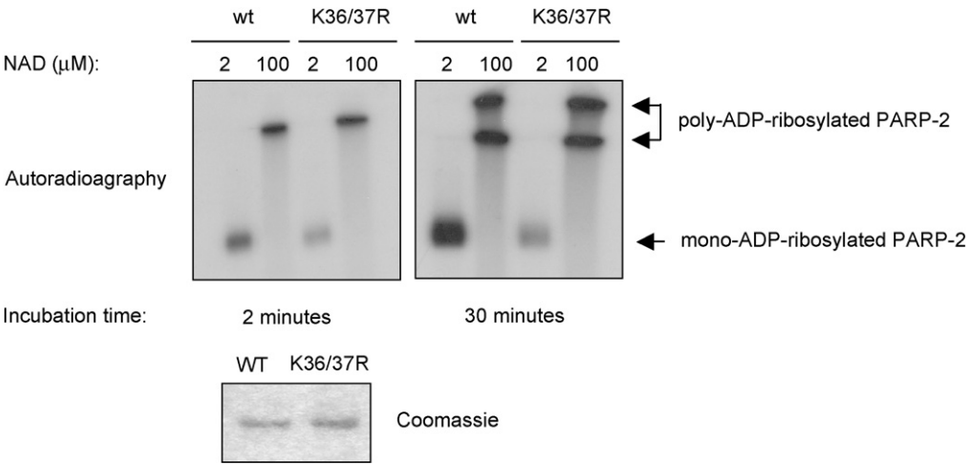

Fig. 5. Lysines 36- and 37 are important for auto-mono-ADP-ribosylation. Wild type (wt) PARP-2 and the mutant K36/37R were expressed in *Sf21* insect cells and purified in the presence of ethidium bromide to avoid copurification of DNA or RNA. Purified proteins were then used for *in vitro* ADP-ribosylation assays in the presence of 2 μM NAD^+, which favors auto-mono-ADP-ribosylation, or in the presence of 100 μM - NAD^+, which stimulates poly-ADP-ribosylation of PARP-2. Products were analyzed by SDS-PAGE and autoradiography, inputs were controlled by Coomassie staining. The lower band reflects the mono-ADP-ribosylated PARP-2. The two upper bands represent auto-poly-ADP-ribosylated forms of PARP-2, as indicated.

of PARP-2 (Supplementary Fig. 2), suggesting that the acetylation of PARP-2 is rather stable compared to the described acetylation of PARP-1 (Hassa et al., 2005). Lysines 36 and 37 are located in a NLS of the proposed DNA binding domain of PARP-2, and mutation of these sites altered the subcellular localization of the protein. Recently, we identified lysine 36 as a major residue required for PARP-2 nuclear localization (manuscript submitted). Since both PCAF and GCN5L localize to the nucleus (Santos-Rosa et al., 2003 and Supplementary Fig. 5), PARP-2 is most likely only acetylated once translocated into the nucleus.

Interestingly, acetylation of PARP-2 was detectable without treatment of cells with a particular stimulus in contrast to PARP-1's acetylation, which was previously described to be induced by TNFα (Hassa et al., 2005). Remarkably, among the different lysine residues found in the domain between aa 1–91, the two identified PCAF/GCN5L target lysines 36 and 37 are conserved between mouse and human, suggesting that regulation by acetylation might also be important for human PARP-2.

Our experiments support the notion that acetylation is directly influencing the DNA binding and enzymatic activity of PARP-2. However, we cannot exclude the possibility that the observed effects were due to impaired dimerization of PARP-2. As described for PARP-1, dimerization of PARP-2 could also be crucial for its DNA binding and enzymatic activity. Moreover, PARP-2 and PARP-1 were recently described to form stable heterodimers within the cell (Schreiber et al., 2002). It is quite possible that the ability of PARP-1 to recruit p300/CBP (Hassa et al., 2005) and the ability of PARP-2 to recruit PCAF/GCN5L acetyltransferases provides this heterodimer with the capability to modulate chromatin structure by alternative interactions with multiple complexes.

Analysis by the Lineweaver-Burk plot estimated a Km of 130 μM for mPARP-2, which represents an affinity for NAD^+ 2.6-fold lower than hPARP-1 (50 μM) (Ame et al., 1999). Even though the enzymatic activity of PARP-2 was overall shown to be lower than that of PARP-1, PARP-2's activity was not strictly dependent on, but enhanced by DNA (Ame et al., 1999). For PARP-1, the ADP-ribose acceptor sites were proposed to be mainly located in the DNA binding and automodification domain. Although these domains are absent in PARP-2, automodification takes place efficiently, indicating that this mode of regulation has also been conserved. Auto-mono-ADP-ribosylation of the PARP-2 mutant K36/37R was substantially reduced under the tested conditions. The reduced auto-mono-ADP-ribosylation could be explained either by a reduced ability of the mutant to dimerize, which would affect *trans*-ADP-ribosylation, or by a reaction mechanism in which lysines K36 and K37 serve as acceptor sites for auto-mono-ADP-ribosylation of PARP-2. Mono-ADP-ribosylation of lysines is believed to occur via the reaction of free ADP-ribose with lysines through Schiff bases (Cervantes-Laurean et al., 1996, 1993; Jacobson et al., 1997 and reviewed in (Hassa et al., 2006). An important general function of acetylation might be to inhibit Schiff base formation of lysines with ADP-ribose, thus representing an interesting possibility of cross-talk between acetylation and ADP-ribosylation of PARP-2 or other PARP family members *in vivo*.

A major general caveat regarding the investigation of PARP-2 functions *in vivo* is the extremely low expression level of endogenous PARP-2. Thus, investigation of the functional consequences of acetylation of endogenous PARP-2 remains a future task.

Interestingly, the K36/37R PARP-2 mutant was still able to synthesize linear and branched poly-ADP-ribose polymers at 100 μM NAD^+ concentrations (Fig. 5). Whether this modification represents the non-covalent binding of formed poly-ADP-ribose polymers to PARP-2 or the existence of other amino acid residues onto which polymers are covalently attached needs to be further addressed. Again these might be other lysine residues, or as suggested for PARP-1, additional glutamic acid residues (D'Amours et al., 1999; Hassa et al., 2006).

Taken together, our experiments identified PARP-2 as substrate for acetylation *in vitro* and *in vivo*. Lysines 36 and 37 were found to be important target sites for acetylation and auto-mono-ADP-ribosylation. Acet-ylation of these two residues by PCAF and GCN5L may negatively affect the DNA binding activity of PARP-2 and consequently also its enzymatic activity *in vivo*.

Acknowledgments

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Appendix A. Supplementary data

Supplementary data associated with this article can be found in the online version, at doi:10.1016/j.biocel.2008.03.008.

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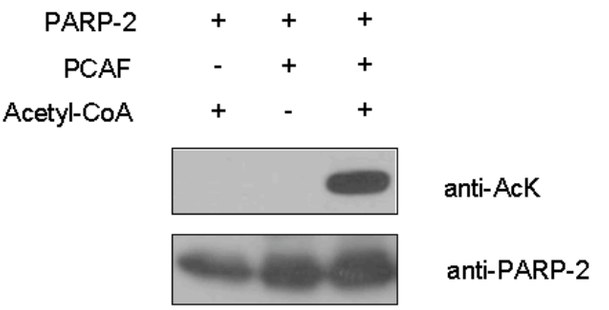
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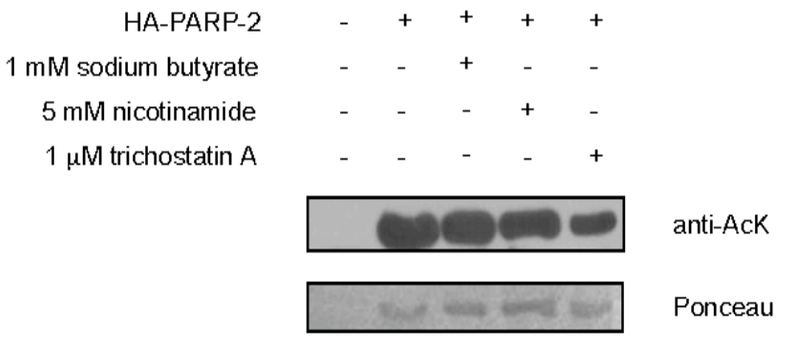
Acetyl-CoA + - +
anti-AcK
anti-PARP-2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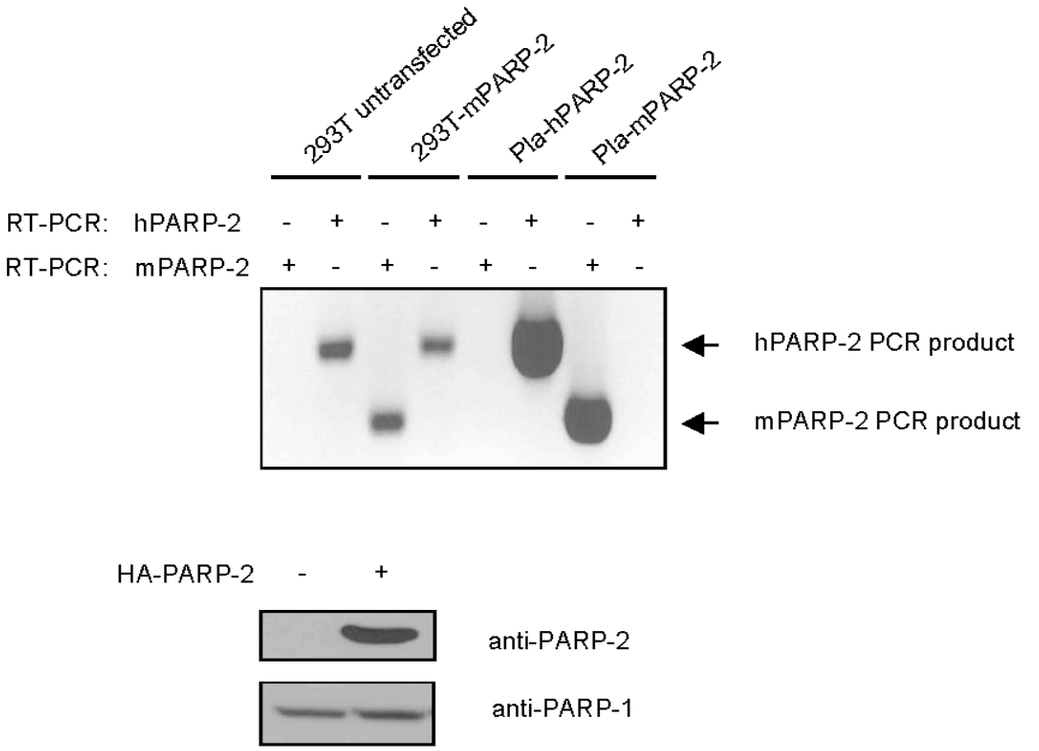
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Pla-hPARP-2
Pla-mPARP-2
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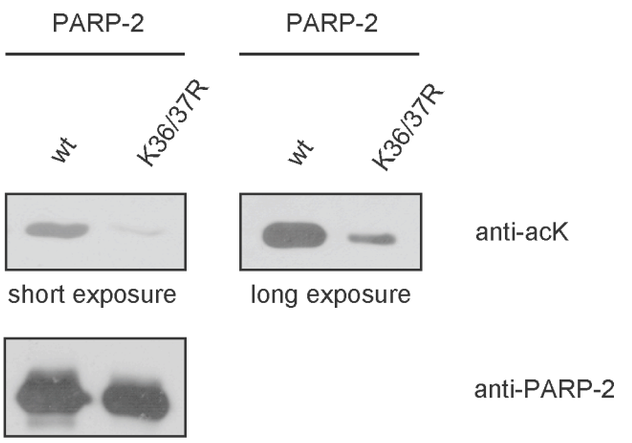
HA-PARP-2 - +
anti-PARP-2
anti-PARP-1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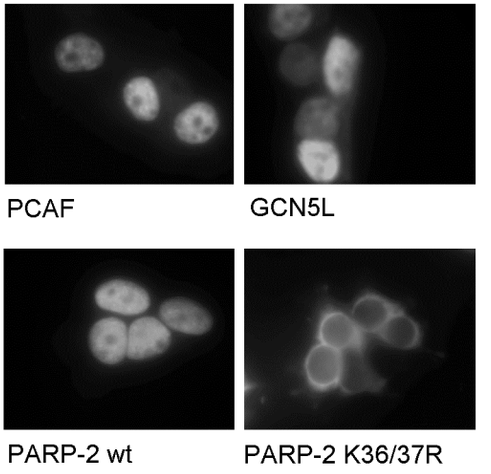
long exposure
anti-PARP-2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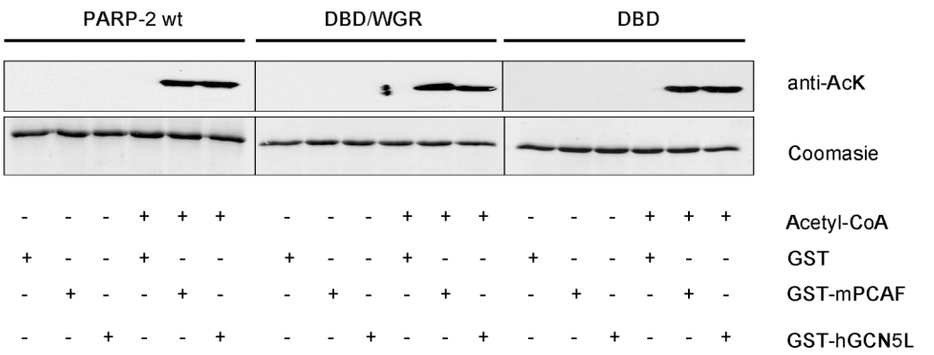
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Importin alpha binding and nuclear localization of PARP-2 is dependent on lysine 36, which is located within a predicted classical NLS

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Abstract

Background: The enzymes responsible for the synthesis of poly-ADP-ribose are named poly-ADP-ribose polymerases (PARP). PARP-2 is a nuclear protein, which regulates a variety of cellular functions that are mainly controlled by protein-protein interactions. A previously described non-conventional bipartite nuclear localization sequence (NLS) lies in the amino-terminal DNA binding domain of PARP-2 between amino acids 1–69; however, this targeting sequence has not been experimentally examined or validated.

Results: Using a site-directed mutagenesis approach, we found that lysines 19 and 20, located within a previously described bipartite NLS, are not required for nuclear localization of PARP-2. In contrast, lysine 36, which is located within a predicted classical monopartite NLS, was required for PARP-2 nuclear localization. While wild type PARP-2 interacted with importin α3 and to a very weak extent with importin α1 and importin α5, the mutant PARP-2 (K36R) did not interact with importin α3, providing a molecular explanation why PARP-2 (K36R) is not targeted to the nucleus.

Conclusion: Our results provide strong evidence that lysine 36 of PARP-2 is a critical residue for proper nuclear targeting of PARP-2 and consequently for the execution of its biological functions.

Background

Poly-ADP-ribosylation reactions occur both in multi- and unicellular organisms and play a major role in a wide range of biological processes, such as maintenance of genomic stability, transcriptional regulation and cell death (reviewed in [1,2]). The enzyme responsible for the synthesis of poly-ADP-ribose was named poly-ADP-ribose polymerase (PARP) (reviewed in [1,2]). For a long time, PARP-1 was thought to be the only enzyme with poly-ADP-ribosylation activity in mammalian cells; however, primary cells derived from *parp-1* knockout mice can still synthesize poly-ADP-ribose polymers after DNA damage [3]. This led to the identification of five novel poly-ADP-ribosylating enzymes, indicating that PARP-1 belongs to a family of at least six members ([4-6] and reviewed in [1,2]). PARP-2 and PARP-1 can homo- and heterodimer-

ize and display partially redundant functions as indicated by the embryonic lethality of the *parp1-parp2*-double gene disruption ([7] and reviewed in [8]).

Mouse PARP-2 was described as a 66 kDa nuclear protein with poly-ADP-ribosylating activity [9]. The amino-terminal region of PARP-2 (aa 1–90), containing the DNA binding SAP domain, has no significant homology with any other PARP [1]. However, it is rich in basic amino acids (27% Lys or Arg), which are likely to be involved in DNA binding (reviewed in [1]). On the other hand, these basic residues could be involved in the nuclear and/or nucleolar targeting of the protein [10]. Previous studies suggested that the nuclear localization signal (NLS) of mPARP-2 is indeed located in the amino-terminal part between aa 1–69 of the protein [9,11]. Meder et al. postulated a bipartite NLS for PARP-2, but did not provide further experimental evidences to support their hypothesis [11]. Interestingly, the amino-terminal region of human and mouse PARP-2 shows higher sequence variability compared to the highly conserved carboxy-terminal catalytic region (62% identity between the amino-terminus of mPARP-2 and hPARP-2). In cells, PARP-2 has been described to regulate different processes via protein-protein interactions mediated by its amino-terminal domain (aa 1–208; reviewed in [1]).

Karyopherins, including both importins and exportins, consitute a conserved family of mobile targeting receptors that mediate the bidirectional trafficking of macromolecules across the nuclear envelope [12,13]. Most karyopherins interact directly with cargo molecules that contain nuclear import and export signals. However, importin α functions as an adaptor that links classical NLS (cNLS)-containing proteins to importin β, which, in turn, docks the ternary complex at the nuclear-pore complex (NPC). The importin α/β heterodimer is predicted to target hundreds of proteins to the NPC and facilitate their translocation across the nuclear envelope [14]. The importin α gene family has undergone considerable expansion during the course of eukaryotic evolution. Whereas the yeast *S. cerevisiae* genome encodes a single importin α, the human genome encodes six genes that fall into three phylogenetically distinct groups [15].

The nuclear targeting signal in the simian virus 40 (SV40) large T antigen was characterized more than 20 years ago [16,17]. Since then, several pathways for nucleocytoplasmic transport have been described, of which the classical nuclear import pathway is the best characterized. cNLSs are typified by either a single cluster of basic amino acids (monopartite NLS) or two clusters of basic amino acids separated by a 10–12 amino acid linker (bipartite NLS). The SV40 large T antigen (PKKKRKV) and nucleoplasmin (KRPAATKKAGQAKKKK) cNLSs are the prototypic monopartite and bipartite cNLS [18,19]. Through alanine scanning of the Myc, monopartite SV40, and artificial bipartite SV40 cNLS, Hodel and colleagues found that the binding affinity of a cNLS for importin measured *in vitro* correlated with the steady state nuclear accumulation and import rate of the corresponding cNLS cargo *in vivo* [20,21].

Here, we demonstrate that lysine 36 in the DNA binding domain (DBD) of PARP-2, which lies within a predicted cNLS motif, is required for complex formation with the importin proteins and subsequent nuclear import of PARP-2.

Results

Lysine 36 and/or lysine 37 of PARP-2 are required for nuclear translocation of PARP-2

Previous experiments with GFP-fusion proteins revealed that the nuclear targeting signal of PARP-2 may be localized between aa 1–69 ([11] and Fig. 1A). This region of the protein was previously postulated to contain a bipartite cNLS; however, this sequence would be an atypical bipartite cNLS as the linker separating the two basic regions is longer than the typical 10–12 amino acid linker. This region does contain a predicted monopartite cNLS that closely matches the canonical SV40 cNLS sequence. To assess whether these sequences are important for nuclear translocation of PARP-2, mutant forms of PARP-2, K19/20R, K36/37R, and K19/20/36/37R, were generated by replacing the lysine residues with arginine residues to maintain the positive charge of the amino acids (Fig. 1B). To exclude the possibility that these amino acid changes altered the stability of the mutated PARP-2, wild type and all mutant forms were expressed as HA-tagged proteins in 293T cells and detected by immunoblot using an anti-HA antibody (Fig. 1C). Immunoblot analysis revealed that all mutants were expressed at a level comparable to wild type PARP-2.

The PARP-2 mutants were transiently transfected and localization was assayed by microscopy of PARP-2 proteins. While wild type PARP-2 and the K19/20R mutant localized in the nucleus, the K19/20/36/37R and K36/37R mutants exclusively localized in the cytoplasm (Fig. 2A). To investigate whether substitution of K36 and K37 with other amino acids altered the localization of PARP-2, similar experiments were repeated with different amino acid susbstitutions. Overexpression of PARP-2 with K19/20, K36/37 or all four residues mutated to glutamate or methionine showed that K → E or K → M substitution of K36/37, but not of K19/20 altered the localization of PARP-2 to a similar extent as the K → R substitution (Fig. 2B and 2C), suggesting that K36 and/or K37 are required for the nuclear translocation of PARP-2, whereas K19 and K20 did not seem to play a role in this process.

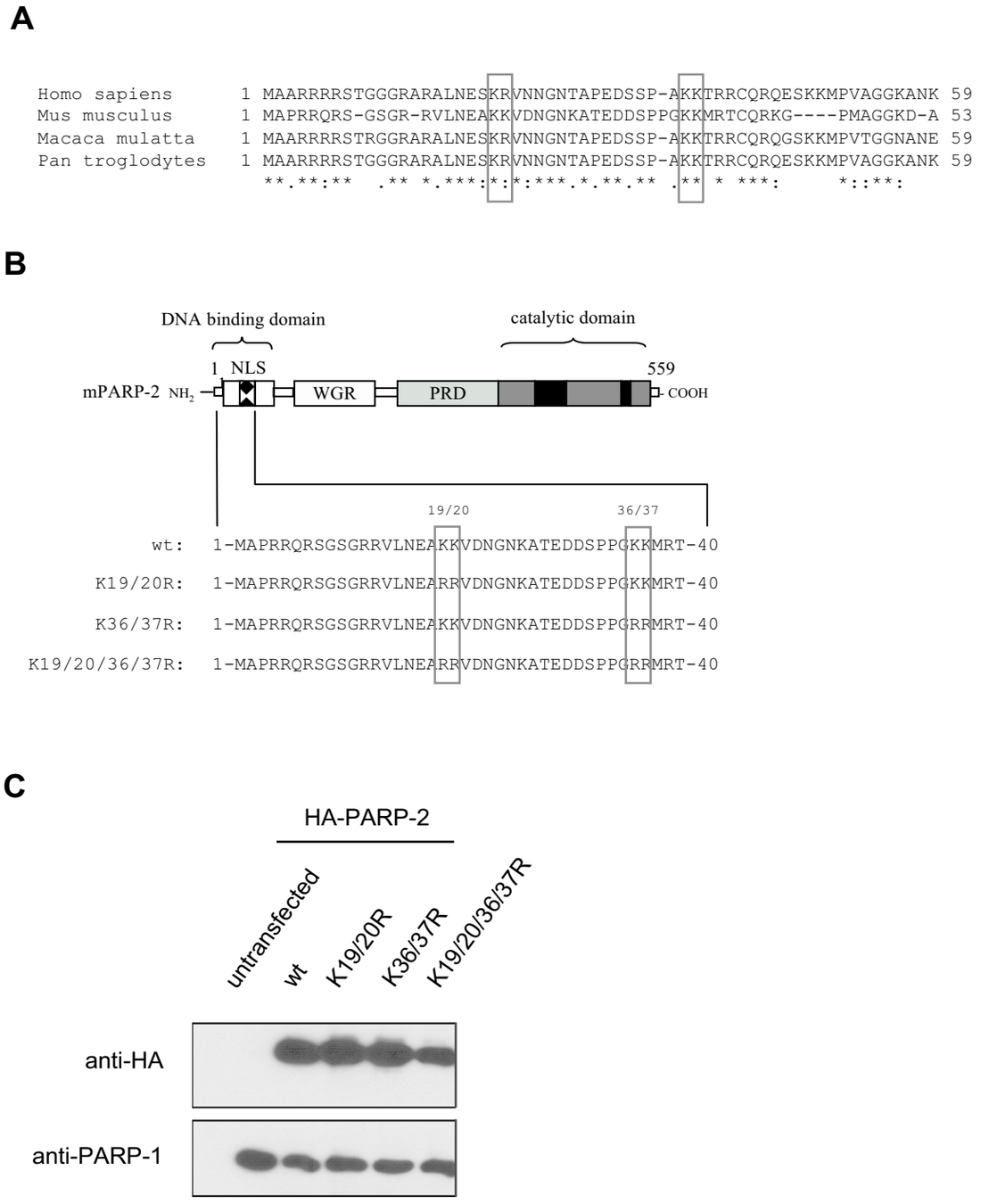

Figure 1

The putative NLS of PARP-2 contains several conserved lysine residues. **A)** Three lysines in the putative NLS of PARP-2 are conserved between different mammalian species. Sequences were obtained from NCBI and alignments were performed using ClustalW2. **B)** Schematic illustration of PARP-2 K → R mutant proteins used in this study: K19, K20, K36 and K37 were changed to arginine using site-directed mutagenesis. Double and quadruple mutants were generated. **C)** HA-tagged wild type (wt) PARP-2 or the indicated double or quadruple mutants were expressed in HEK293T cells and expression was analyzed by western blot using a monoclonal anti-HA antibody. 100 μg of whole cell extracts were used, endogenous PARP-1 levels served as loading control.

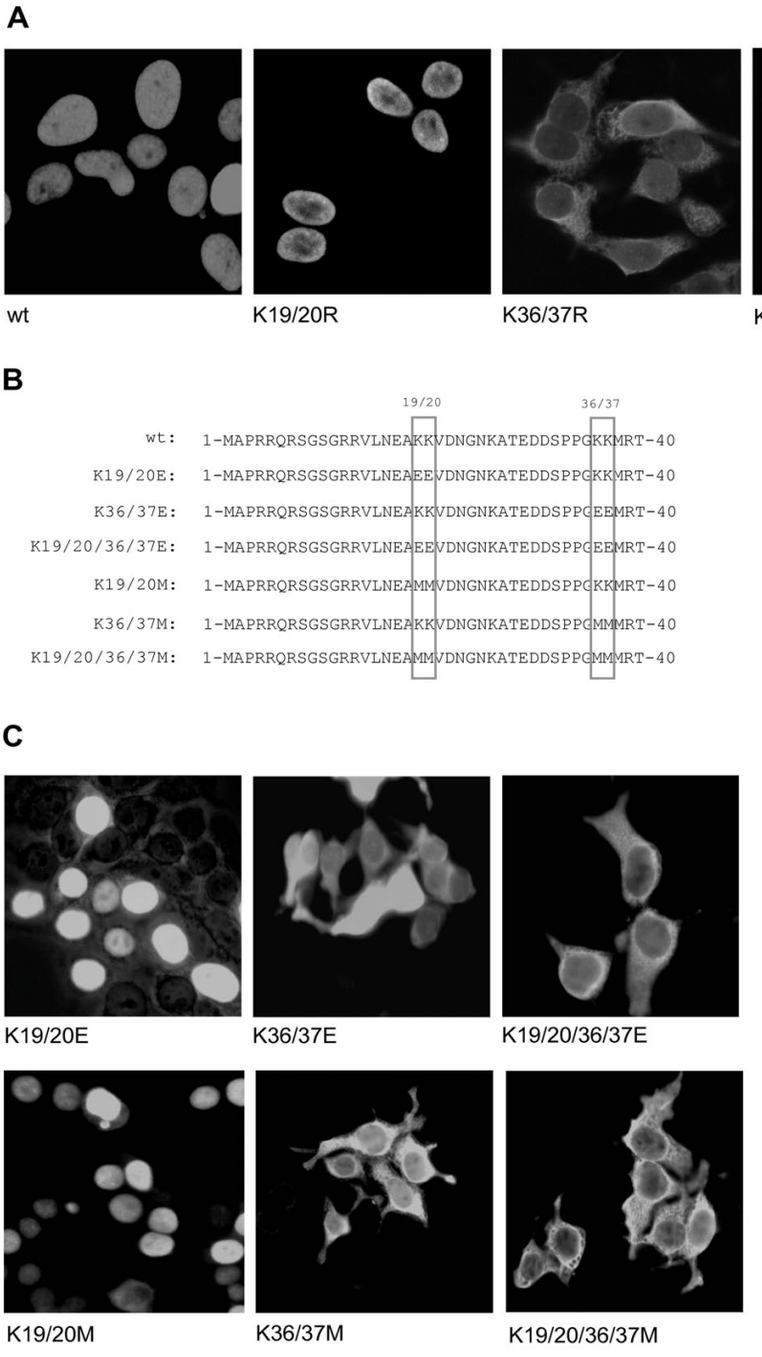

Figure 2

Lysine 36 and/or lysine 37 of PARP-2 are required for nuclear localization. **A)** HEK293T cells were transfected with HA-tagged wild type (wt) PARP-2 or with the indicated mutants. Cells were fixed with methanol for subsequent detection of HA-tagged proteins by immunofluorescence using an anti-HA antibody and a FITC-conjugated anti-mouse antibody. Representative confocal images are presented. **B)** Lysines 19, 20, 36 and 37 of PARP-2 were changed to glutamic acid or methionine as indicated. **C)** The nuclear localization is independent of the charge but dependent on the structure of the NLS. As for Fig. 2A, HEK293T cells were transfected with HA-tagged wild type (wt) PARP-2 or with the indicated K → E and K → M mutants and overexpressed proteins were detected as described for Fig. 2A. Representative confocal images are presented.

Leptomycin B does not change cellular localization of PARP-2 mutant K36/37R

Next, we investigated whether the cytoplasmic localization of the mutated PARP-2 protein K36/37R is caused by an abrogated nuclear import or by an accelerated nuclear export of a transiently nuclear localized PARP-2 mutant. Cells were transfected with wild type or mutant PARP-2 and subsequently treated with Leptomycin B (LMB), a well-characterized inhibitor of CRM-1-mediated nuclear export [22-24]. Treatment with LMB did not induce any changes in the cellular localization of the PARP-2 mutant K36/37R (Fig. 3), indicating that K36 and/or K37 are more likely to impact nuclear import of PARP-2 than a classical NES-mediated export process.

Lysine 36 but not lysine 37 of PARP-2 is required for nuclear localization of PARP-2

In order to further investigate the requirement for K36 and K37 in the nuclear localization of PARP-2, K → R single mutants were created at each position. Interestingly, similar experiments performed with the wild type and the single mutants of PARP-2 possessing K36R and K37R substitutions revealed, that both mutants were stably expressed at levels comparable to wild type PARP-2 and that only lysine 36 was important for the nuclear accumulation of PARP-2 (Fig. 4A and 4B). In contrast to earlier reports [11], no nucleolar staining was observed under the tested conditions. These experiments identified K36 as an important residue for the nuclear localization of PARP-2 *in vivo*.

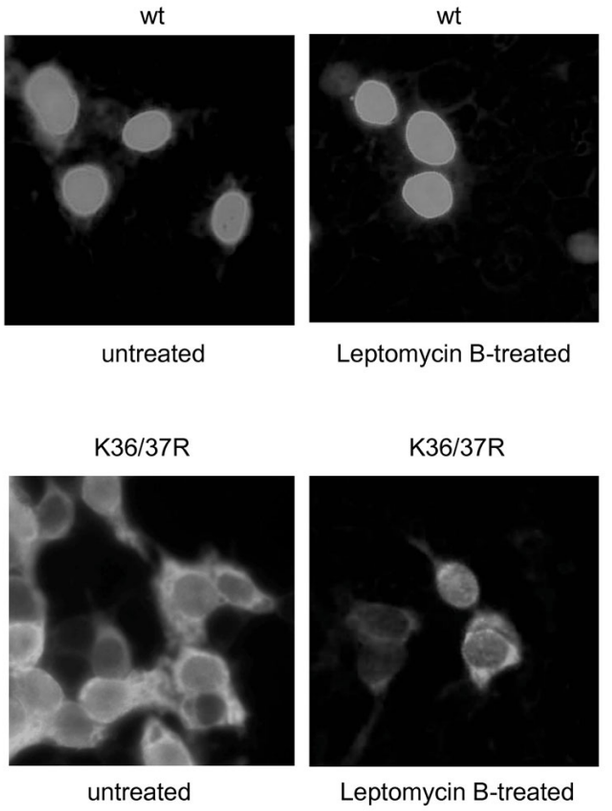

Figure 3
Leptomycin B does not alter localization of the PARP-2 mutant K36/37R. HEK293T cells were transfected with HA-tagged wild type (wt) PARP-2 (upper panel) or with the mutant K36/37R (lower panel) and treated with Leptomycin B (LMB) to inhibit nuclear export, followed by immunofluorescence as described in Fig. 2. Representative images are presented.

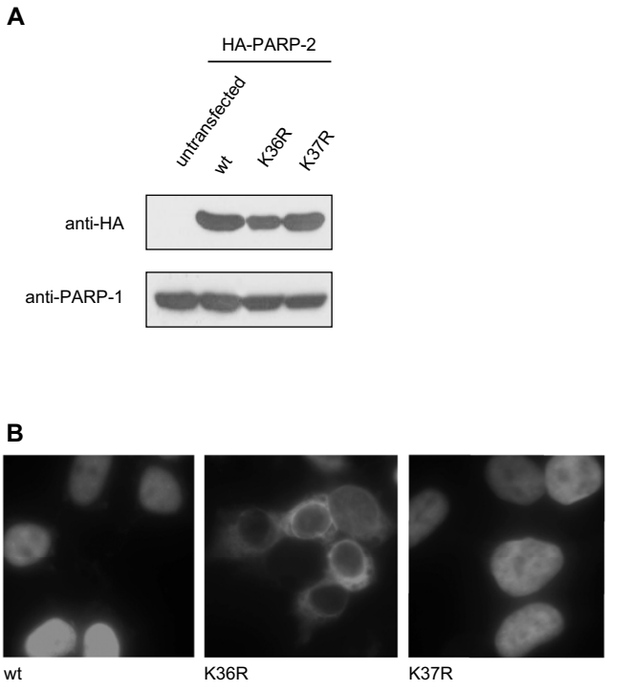

Figure 4
Lysine 36 but not lysine 37 of PARP-2 is critical for nuclear localization. **A)** HA-tagged wild type (wt) PARP-2 or the indicated single mutants were expressed in HEK293T cells and expression was analyzed by western blot using a monoclonal anti-HA antibody. 50 μg of whole cell extracts were used, endogenous PARP-1 levels served as loading control. **B)** HEK293T cells were transfected with HA-tagged wild type (wt) PARP-2 or with the PARP-2 mutants K36R and K37R. HA-tagged proteins were detected by immunofluorescence as described for Figure 2A. Representative images are presented.

Lysine 36 is important for binding to importin α3

One possibility to confirm the functional cNLS targeting sequence is to perform interaction studies with the classical NLS import receptor, importin α. In order to test whether PARP-2 interacts with importin α, we performed GST pull-down experiments with different recombinant purified GST-fusion proteins of human importin α (α1, α3, α5 and α7; Fig. 5A) and cell extracts containing overexpressed wild type or different mutated PARP-2 proteins. PARP-2 was detected in the bound fraction following the pull-down assay by western blot analysis. Wild type PARP-2 formed a complex with importin α3 and to a very weak extent also with importin α1 and importin α5, but not with importin α7 (Fig. 5B). Experiments with purified wild type and mutated PARP-2 (K36/37R, K36R and K37R) revealed that the mutant proteins K36/37R and K36R did not bind importin α3, while the K37R mutant did bind, suggesting that K36 is a critical residue of PARP-2 essential for its interaction with importin α3 and its nuclear translocation (Fig. 5C and 5D).

Discussion

PARP-2 regulates different cellular functions. Here, we provide both biochemical and functional evidence that substitution of lysine residue 36 efficiently inhibits localization of PARP-2 to the nucleus. Functional analyses revealed that lysine 36 is important for complex formation with importin α3.

Lysine residues are central components of classical NLS motifs (reviewed by [25]) as their positive charge mediates the interaction with importin receptors [26]. Here we provide evidence that K36 of PARP-2 is an important residue required for the nuclear translocation of PARP-2 and for complex formation with importin α3, as mutation of this residue was sufficient to disrupt association with the import machinery and subsequently alter PARP-2 nuclear localization. Interestingly, lysine 36 is conserved between mouse and human PARP-2, suggesting that the described findings might also apply for the human counterpart. Together, our data indicate that the nuclear import of human and murine PARP-2 is mediated by a conserved classical monopartite NLS but not through a bipartite NLS as previously proposed [11].

The formation of the importin-α/β-cNLS cargo ternary complex is the first step in the nuclear transport of hundreds of different nuclear proteins, and, as such, is tightly regulated [15]. The relationship of importin α/β with its cNLS cargo is by necessity bipolar, because it forms highly selective and tight complexes in the cytoplasm and then switches to an extremely low affinity state in the nucleus to release the cargo. When importin α is not bound to importin β, an autoinhibitory sequence within the amino-terminal domain apparently interacts with the NLS-binding pocket [27]. This interaction is not exceptionally strong because cNLS cargos can still bind to importin α in the absence of importin β, albeit with significantly lower affinity. The order of importin α binding to cNLS cargo and importin β is not known. The observed lack of importin α3 binding by the PARP-2 mutant (K36R) clearly indicates that this lysine is required for the interaction with importin α and subsequently for nuclear translocation.

Recently, it has become evident that importin α receptors have independent roles in the assembly of macromolecular structures. Genetic analyses of yeast importin α mutants identified several alleles that confer defects in chromosome and nuclear segregation, altered mitotic spindle structure and deficits in the ubiquitin-mediated protein degradation pathway [28-31]. Mechanistic studies on the roles of importin αs in mitosis, spindle assembly and nuclear envelope biogenesis point more directly to activities which are independent of the housekeeping roles of importin α in nuclear transport. The observed interaction of PARP-2 with importin α might thus not only be important for its nuclear translocation but might have an additional physiological function in maintaining the integrity of the genome. Inactivation of the *parp-2* gene in mice revealed that PARP-2 may be involved in the surveillance and maintenance of genome integrity, indicated by the sensitivity of these mice to ionizing radiation [7].

Others have reported that PARP-2 is enriched within the whole nucleolus and partially colocalizes with the nucleolar factor nucleophosmin/B23 [11]. Using partial cDNA fragments in-frame with the carboxy-terminus of EGFP the authors described a putative nuclear localization signal and a nucleolar localization signal within the amino-terminal domain of PARP-2 (aa 1–69). Our studies revealed that overexpressed PARP-2 was only found equally distributed in the nucleus, but in contradiction to this previous report, was never observed in the nucleolus of the cell. This discrepancy could be explained by the different experimental approaches used. Meder et al. studied the nucleolar localization of PARP-2 with GFP-fusion proteins, while our studies were performed with non-GFP tagged full-length proteins. Remarkably, PARP-1 nucleolar accumulation was not observed when endogenous or overexpressed PARP-1 localization was analyzed by a conventional immunofluorecence protocol as described in Methods using specific anti-PARP-1 antibodies (data not shown). Only applying the fixation protocol described in Meder et al. [11], which led to the decomposition of the cell and loss of cytoplasm, revealed the reported nucleolar staining of PARP-1, suggesting that the fixation protocol influences the nucleolar localization of proteins or the detection of proteins within the nucleolus.

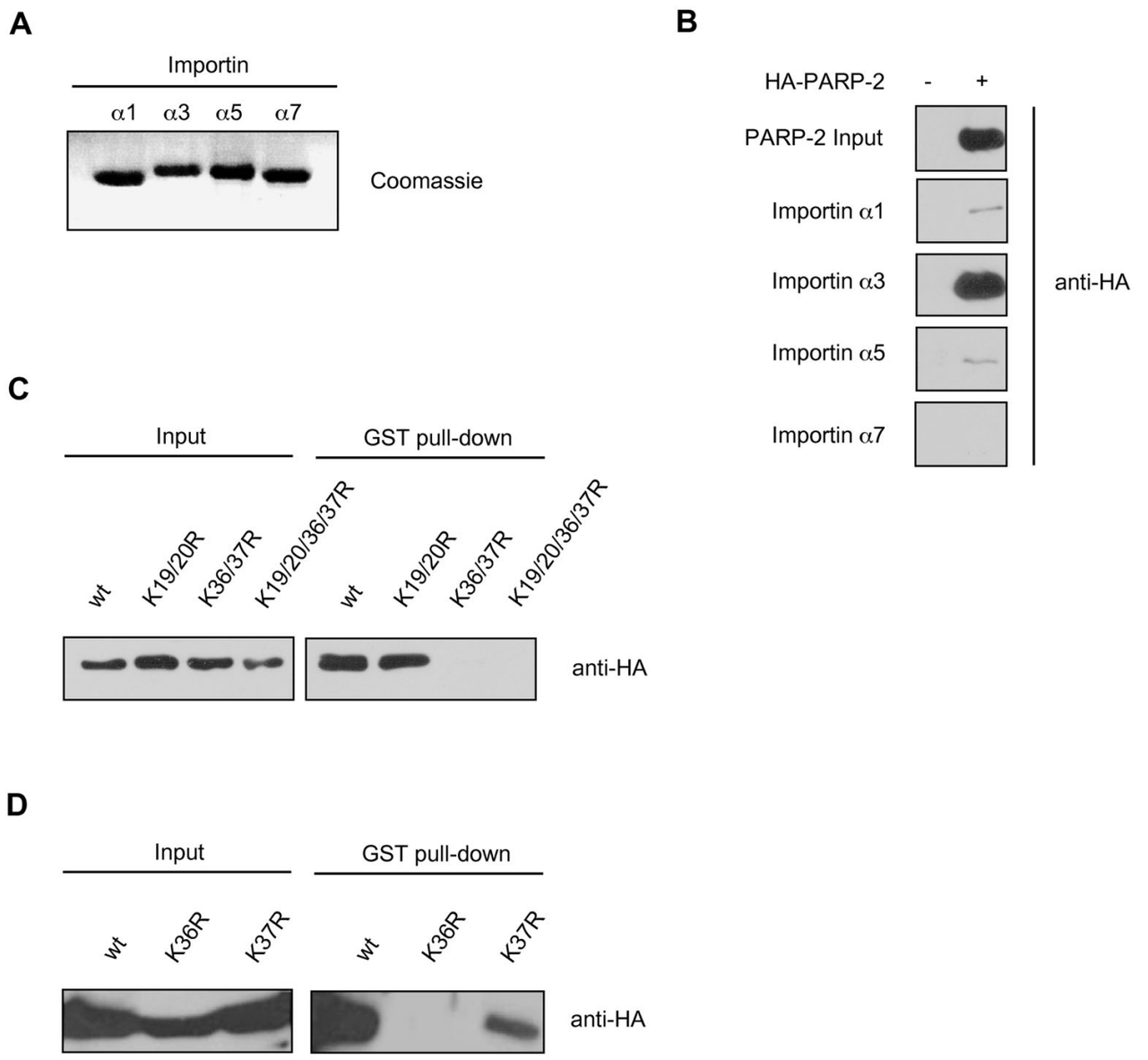

Figure 5

Lysine 36 of PARP-2 is necessary for the binding of PARP-2 to importin α3. A) Importins α1, α3, α5 and α7 were expressed as GST-fusion proteins in *E. coli* and purified with Glutathione Sepharose 4B beads. Expression was checked by SDS-PAGE followed by Coomassie staining. **B)** PARP-2 binds mostly to importin α3 and to a lower extent to importin α1 and α5. Purified GST-importins were incubated with whole cell extracts from HEK293T cells, either untransfected or transfected with wild type HA-PARP-2, then western blot analysis was performed using an anti-HA antibody. **C)** Lysines 36/37 are required for the binding of PARP-2 to importin α3. Purified GST-importin α3 was incubated with whole cell extracts from HEK293T cells transfected with either wild type (wt) HA-PARP-2 or with the indicated double and quadruple mutants. Proteins were separated by SDS-PAGE and analyzed by western blot using an anti-HA antibody. **D)** Lysine 36 but not lysine 37 is required for the binding of PARP-2 to importin α3. GST-importin α3 was bound to Glutathione Sepharose 4B and incubated with whole cell extracts from HEK293T cells expressing either wild type (wt) PARP-2 or the indicated single mutants. PARP-2 bound to importin α3 was detected using an anti-HA antibody.

Recently, acetylation of lysine residues by histone acetyltransferases (HATs), such as p300/CBP (CREB-binding protein) and PCAF (p300/CBP-associated factor), has been proposed as a new mechanism for modulating cellular localization [32-36]. HATs trigger the transfer of an acetyl group from acetyl coenzyme A to the epsilon-amino group of a lysine residue not only on core histones but also on about 40 transcription factors and on more than 30 other proteins [37]. We recently published that both lysines 36 and 37 of PARP-2 are indeed acetylated *in vitro* and *in vivo* and that acetylation influences both DNA binding and auto-ADP-ribosylation of PARP-2 [38].

Conclusion

Taken together, our results provide evidence that PARP-2 accumulates in the nucleus and that lysine 36, which is located within a monopartite cNLS, is important for binding of PARP-2 to importin α3 and for the nuclear translocation of PARP-2.

Methods

Plasmids

Mammalian expression vectors for wild type PARP-2 and all mutants used in this study were obtained by cloning the corresponding PCR products into pphCMV-HA. PARP-2 mutants were generated by a site directed mutagenesis procedure and confirmed by sequencing. Bacterial expression vectors for human GST-importins α1, α3, α5 and α7 were provided by Dr. Riku Fagerlund (Departments of Viral Diseases and Immunology and Epidemiology and Health Promotion, National Public Health Institute, FIN-00300, Helsinki, Finland, [39]).

Expression and purification of recombinant proteins

GST-tagged importins were expressed in *E. coli* strain BL21-D3-Gold. All purified proteins were analyzed by Coomassie staining and confirmed by western blot analysis using the corresponding antibodies.

Cell culture and transient transfections, treatment with LMB and immunofluorescence

HEK293T cells were grown in Hepes-buffered DMEM-Glutamax-I (Invitrogen) containing 4.5 g/L glucose and 10% FCS US/certified (Invitrogen) and supplemented with 50 U/ml penicillin, 50 μg/ml streptomycin (Invitrogen) and MEM non-essential amino acids (MEM NEAA, Invitrogen). Cells were transfected using calcium phosphate procedures as described in [40]. For the experiments with Leptomycin B (LMB), cells were treated with a final concentration of 20 ng/ml LMB for 4–16 hrs. For detection of overexpressed proteins by immunofluorescence, HEK293T cells were fixed for 10 minutes in ice-cold 100% methanol in the absence of detergents and unspecific binding sites were blocked with 2% BSA/0.1% Triton X-100 prior to staining with primary and FITC-conjugated secondary antibodies in the presence of 2% BSA/0.1% Triton X-100 according to the manufacturer's protocol (Covance) using confocal (Leica SP2, 40× oil-immersion, NA 1.25, zoom-in) or standard fluorescence microscopy (Olympus Mx51, 100× oil-immersion, NA 1.3).

Western blot analysis and antibodies

Western blot analyses were performed as described previously [41]. Anti-myc-9E10 (sc-2027) antibodies were obtained from Santa Cruz Biotechnology, anti-HA (MMS-101P) was obtained from COVANCE. Antibodies against mouse PARP-1 and PARP-2 were generated in house (the generation of antibodies against mouse PARP-1 has been described previously [42,43]).

In vitro *interaction and GST pull-down assays*

Purified recombinant proteins fused to GST were bound to Glutathione Sepharose 4B according to the manufacturer's protocols (Amersham Biosciences). GST pull-down assays were performed as described previously [41,42]. GST pull-down-buffers contain: 50 mM Tris [pH 8.0], 150 mM NaCl, 0.5% NP-40, 0.5 mM DTT, 1 mM PMSF, 100 μM bestatin, 3 μM pepstatin A, 5 μM leupeptin. Bound proteins were dissolved by SDS PAGE and subsequently analyzed by western blot.

Authors' contributions

SSH carried out the molecular studies and drafted the manuscript. MA carried out the molecular studies with the single PARP-2 mutants and drafted the figures and helped to draft the manuscript. POH participated in the design of the study, carried out the site directed mutagenesis and helped to draft the mansucript. TV participated in the design of the study. MF purified recombinant proteins. MOH conceived the study and participated in its design and coordination and helped to draft the manuscript. All authors read and approved the final manuscript.

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Sumoylation of poly(ADP-ribose) polymerase 1 inhibits its acetylation and restrains transcriptional coactivator function

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ABSTRACT **Poly(ADP-ribose) polymerase 1 (PARP1) is a chromatin-associated nuclear protein and functions as a molecular stress sensor. At the cellular level, PARP1 has been implicated in a wide range of processes, such as maintenance of genome stability, cell death, and transcription. PARP1 functions as a transcriptional coactivator of nuclear factor κB (NF-κB) and hypoxia inducible factor 1 (HIF1). In proteomic studies, PARP1 was found to be modified by small ubiquitin-like modifiers (SUMOs). Here, we characterize PARP1 as a substrate for modification by SUMO1 and SUMO3, both *in vitro* and *in vivo*. PARP1 is sumoylated at the single lysine residue K486 within its automodification domain. Interestingly, modification of PARP1 with SUMO does not affect its ADP-ribosylation activity but completely abrogates p300-mediated acetylation of PARP1, revealing an intriguing crosstalk of sumoylation and acetylation on PARP1. Genetic complementation of PARP1-depleted cells with wild-type and sumoylation-deficient PARP1 revealed that SUMO modification of PARP1 restrains its transcriptional coactivator function and subsequently reduces gene expression of distinct PARP1-regulated target genes. Messner, S., Schuermann, D., Altmeyer, M., Kassner, I., Schmidt, D., Schär, P., Müller, S., and Hottiger, M. O. Sumoylation of poly(ADP-ribose) polymerase 1 inhibits its acetylation and restrains transcriptional coactivator function. *FASEB J.* 23, 000–000 (2009). www.fasebj.org**

Key Words: NAD · SUMO · hypoxia · PARP-1

Poly(ADP-ribose) polymerase 1 (PARP1) is an abundant nuclear chromatin-associated multifunctional enzyme found in higher eukaryotes that belongs to a family of 5 "*bona fide*" PARP enzymes (1). PARP1 has an amino-terminal DNA-binding domain (DBD) containing 3 zinc finger motifs, as well as a central automodification domain (AMD), which functions as a target of direct covalent automodification. The carboxyl-terminal catalytic domain polymerizes linear or branched chains of ADP-ribose from the donor nicotinamide adenine dinucleotide (NAD^+). ADP-ribose is mainly attached on PARP1, but also other proteins are modified (2). Together, the DBD and the automodification domain allow PARP1 to interact with genomic DNA and chromatin. Although originally characterized as a key factor in DNA single strand-break repair, a wealth of studies over the past decade have demonstrated a role of PARP1 in the regulation of gene expression under basal, signal-activated, and stress-activated conditions (1, 3). Recent studies have highlighted the role of PARP1 in distinct modes of transcriptional regulation and provided novel insight into the cellular signaling systems that interface with PARP1 in the nucleus (4).

The basal enzymatic activity of PARP1 is very low, but it is stimulated dramatically under conditions of cellular stress (2, 3). Activation of PARP1 results in the synthesis of poly(ADP-ribose) (PAR) from NAD^+ and the release of nicotinamide as a reaction by-product (1). Following PARP1 activation, intracellular PAR levels can rise 10- to 500-fold (1), caused by a mechanism that remains to be resolved. Very recently, we identified 3 lysine residues in the automodification domain of PARP1 as acceptor sites for auto-ADP-ribosylation (5). PARP1 is the main acceptor for poly(ADP-ribosyl)ation *in vivo*, and automodification of PARP1 abolishes its affinity for NAD^+ and DNA (5). Remarkably, the same 3 ribosylated lysines (K498, K521, K524) were previously identified as targets for acetylation by the histone acetyltransferase p300 (6). Acetylation of PARP1 has been reported to be important for its transactivation activity (6). Recently, we also highlighted the role of PARP1 as a transcriptional coactivator of hypoxia inducible factor 1-α (HIF1-α). On hypoxic induction of cells, PARP1 was shown to interact with HIF1-α and to regulate the transcriptional activity of HIF1-α-dependent genes (7).

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A macrodomain-containing histone rearranges chromatin upon sensing PARP1 activation

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Poly-ADP-ribosylation is a post-translational modification catalyzed by PARP enzymes with roles in transcription and chromatin biology. Here we show that distinct macrodomains, including those of histone macroH2A1.1, are recruited to sites of PARP1 activation induced by laser-generated DNA damage. Chemical PARP1 inhibitors, PARP1 knockdown and mutation of ADP-ribose–binding residues in macroH2A1.1 abrogate macrodomain recruitment. Notably, histone macroH2A1.1 senses PARP1 activation, transiently compacts chromatin, reduces the recruitment of DNA damage factor Ku70–Ku80 and alters γ-H2AX patterns, whereas the splice variant macroH2A1.2, which is deficient in poly-ADP-ribose binding, does not mediate chromatin rearrangements upon PARP1 activation. The structure of the macroH2A1.1 macrodomain in complex with ADP-ribose establishes a poly-ADP-ribose cap-binding function and reveals conformational changes in the macrodomain upon ligand binding. We thus identify macrodomains as modules that directly sense PARP activation *in vivo* and establish macroH2A histones as dynamic regulators of chromatin plasticity.

Post-translational modifications, ATP-dependent remodelers, histone variants and histone H1 dynamically alter chromatin structure[1]. One of the oldest and least understood post-translational modifications is the poly-ADP-ribosylation (PARylation) of proteins, including histones, a modification that is mediated by poly-ADP-ribose (PAR) polymerases including PARP1 and PARP2 (refs. 2,3). Nuclear PARP1 is the main PAR acceptor, and its activity is induced by stress-response pathways, such as metabolic stress, mitogen-activated protein kinase (MAPK) signaling, DNA breaks[4], promoter–stem-loop and cruciform DNA structures and changes in polyamines, as well as Mg^{2+} and Ca^{2+} concentration[5]. Genetic and biochemical data indicate that PAR has roles in chromatin biology, differentiation and apoptosis[2,3].

PARP1 activation by stress-response pathways motivated the search for proteins that recognize PAR. PAR-binding linear motifs and atypical zinc fingers have been described[6], but no globular protein module—typical of signaling pathways—has been identified. We reported that globular macrodomains, including those in histone macroH2A1.1, bind NAD^+ metabolites related to ADP-ribose (ADPR), a PAR metabolite produced by PAR glycohydrolase (PARG), and *O*-acetyl-ADPR, which is produced by sirtuins[7]. However, slot-blot assays suggested that macroH2A1.1 macrodomains do not bind PAR, whereas archaebacterial Af1521 macrodomains retain PAR[7]. Thus, despite biochemical data implicating macroH2A or its C-terminal macrodomain in PARP1 binding[7–10], there is no evidence to link macrodomains to the binding of ADPR moieties in mono-, oligo- and poly-ADP-ribosylated (*n*-ADP-ribosyl) PARP1. Furthermore, because the splice variant macroH2A1.2 cannot bind ADPR, it might differ in its interaction with *n*-ADP-ribosyl-PARP1. We thus set out to test whether macrodomains recognize nuclear sites in human cells with high PAR metabolism, such as sites where activated PARP1 *n*-ADP-ribosylates target proteins or, alternatively, where PARG mediates the rapid degradation of PAR to ADPR, thus producing a transiently high, local ADPR concentration.

RESULTS

The macrodomain senses PARP1 activation

Distinct mechanisms activate PARP1, many of which do not involve DNA damage. However, to test whether macrodomains recruit to nuclear regions where PAR is generated, accumulates and is subsequently degraded to ADPR, we took advantage of a robust model system that activates PARP1 using pulsed-laser DNA microirradiation, providing a convenient readout for transient PAR accumulation[11,12] within a spatially defined region (the 'laser cut'). We therefore used a 'low' laser power to microirradiate HeLa nuclei expressing fluorescent protein–tagged full-length Af1521 and macroH2A1.1 macrodomain (in the absence of macroH2A's histone fold region; note that HeLa and other proliferating cells do not express detectable amounts

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PARP1 accelerates *Salmonella*-induced gut inflammation

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Short title: PARP1 and *Salmonella*

Author contributions: MA, MB and ME performed experiments; HR helped with microarray data analysis and statistics; MA analyzed gene expression data and drafted the manuscript; WDH and MOH designed and supervised the study.

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Abbreviations: PARP1, poly(ADP-ribose) polymerase 1; NF-κB, nuclear factor-kappaB; IFNγ, interferon gamma; *S*.tm, *Salmonella* Typhimurium; p.i., post infection

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http://www.ncbi.nlm.nih.gov/geo/query/acc.cgi?token=fzaxzcmcgagwazm&acc=GSE19174

Abstract

BACKGROUND & AIMS: The immune system comprises an innate and an adaptive immune response to combat pathogenic agents. The human enteropathogen *Salmonella enterica* serovar Typhimurium invades the intestinal mucosa and triggers an early innate pro-inflammatory host gene response, which results in diarrheal disease.

Several host factors are involved in the acute early response to *Salmonella* infection. Transcription factors and transcription co-regulators have an especially important function, because they are required for the expression and synthesis of pro-inflammatory cytokines, chemokines and adhesion molecules. A central transcription factor involved in inflammation is NF-κB, which requires the nuclear protein PARP1 as co-factor for the expression of some of its target genes. Here, we investigated the role of PARP1 during *Salmonella* infection.

METHODS: To study enterocolitis by *Salmonella* Typhimurium, the streptomycin mouse model system was employed. Histopathologic signs of inflammation and cecum colonization at various time-points after infection of wild type and PARP1 knockout mice were analyzed. PARP1 expression in the gut mucosa was studied by quantitative RT-PCR, Western blot and immunofluorescence. Gene expression profiles of infected and control infected mice in the wild type or PARP1 knockout background were obtained by whole mouse genome arrays and confirmed by quantitative RT-PCR.

RESULTS: PARP1 is expressed in the proliferative zone of cecum crypts and is associated with a faster pro-inflammatory response after *Salmonella* infection. The accelerated PARP1-dependent host response involves higher expression of pro-inflammatory genes, many of which are related to IFNγ signaling, and more severe inflammation with increased infiltration of immune cells.

CONCLUSION: PARP1 facilitates pro-inflammatory gene expression, which accelerates *Salmonella*-induced inflammation.

Keywords: PARP-1; *Salmonella* Typhimurium; colitis; inflammation

Introduction

The innate immune system constitutes the first line of host defense during infection. Innate immunity is therefore crucial for the early recognition of invading pathogens and for the subsequent pro-inflammatory response [1]. Recognition of pathogen-associated molecular patterns (PAMPs) is achieved by pattern recognition receptors (PRRs), such as the family of Toll-like receptors (TLRs) [2]. Upon PAMP recognition, PRRs trigger pro-inflammatory and anti-microbial responses by activating a multitude of intracellular signaling pathways and inducing inflammation-related transcription factors [3]. Ultimately, this results in the expression and synthesis of a broad range of molecules, including cytokines, chemokines, and adhesion molecules, which together orchestrate the early host response to infection and significantly contribute to inflammation.

The intestinal mucosa is constantly exposed to numerous microbes, some of which may be pathogenic. Whereas in the absence of pathogens the healthy intestine is characterized by homeostasis of the immune system with the commensal microbial flora, in the presence of pathogens this homeostasis is disturbed. In such a situation of acute infection, pro-inflammatory responses are triggered by the innate immune system to limit infection.

The human enteropathogen *Salmonella enterica* subspecies 1 serovar Typimurium (*S.* Typhimurium) invades the intestinal mucosa, causes self-limiting gut infection and elicits mucosal inflammation and diarrhea. *Salmonella* infection is a global threat to human health, but the molecular mechanisms underlying *Salmonella*-induced enteric diseases are not sufficiently understood. A mouse model system to study *S.* Typhimurium enteropathogenesis was described several years ago [4]. In this model system, streptomycin-pretreated mice develop severe colitis after infection with serovar Typhimurium, which largely resembles the human infection. The streptomycin-pretreated mouse model has been used frequently as an appropriate and well-established model system to study the mechanisms of pathogenesis and the host responses to acute enteric salmonellosis [5-9].

The protein poly(ADP-ribose) polymerase 1 (PARP1) is an abundant nuclear chromatin-associated enzyme with a variety of cellular functions. PARP1 has been implicated in DNA damage signaling and repair, chromatin remodeling and transcriptional regulation [10]. PARP1 knockout mice are viable and fertile, have normal life span and show no increase in spontaneous tumor frequency [11]. They are, however, protected from tissue injury in various inflammation-related disease models ranging from myocardial infarction, streptozotocin-induced diabetes, to LPS-induced septic shock and arthritis [11-13]. These

phenotypes suggest an important role for PARP1 during inflammation. The protective effects observed in PARP1 knockout animals correlate with the reduced expression of pro-inflammatory cytokines under the control of the inducible transcription factor nuclear factor-kappaB (NF-κB) [14]. In line with these observations, PARP1 was found to function as co-activator for NF-κB-dependent gene expression [15].

Here, we investigated a possible role for PARP1 in Typimurium-induced colitis. Employing the streptomycin mouse model for enterocolitis, we analyzed the impact of PARP1 on the *Salmonella*-triggered innate immune response and early development of cecal inflammation. We demonstrate that PARP1 is strongly expressed in the proliferative zone of the cecum and is associated with a faster pro-inflammatory host response. This accelerated response involves higher expression of pro-inflammatory genes and more severe inflammation with increased infiltration of immune cells. Our findings link, for the first time, PARP1 to *Salmonella*-induced pro-inflammatory gene expression and suggest an important role for PARP1 in timing the host response to enteric *Salmonella* infection.

Materials and Methods

Bacteria

Salmonella Typhimurium (*S.*tm) strains were isogenic derivatives of the naturally streptomycin-resistant wild type strain SL1344 [16]. *S.*tm^{avir} is SL1344, Δ*inv*G [17]. Strains were grown at 37°C in LB (0.3M NaCl) overnight and subcultivated for 4 hours as described before [18].

Mice

Wild type and isogenic PARP1 knockout mice [11] were bred in a C57Bl/6J background. Regular back-crossings were performed to maintain isogenicity. Animals were genotyped by PCR (primer sequences for wild type: 5`-GTTGTGAACGACCTTCTGGG-3` and 5`-CCTTCCAGAAGCAGGAGAAG-3`; primer sequences for PARP1 knockout: 5`-GTTGTGAACGACCTTCTGGG-3` and 5`-GCTTCAGTGACAACGTCGAG-3`). All mice were bred and kept in a specified pathogen free area. Streptomycin pretreated mice (20 mg/animal) were infected by gavage (5 x 10^7 cfu) as published previously [4, 9]. Live bacterial loads in cecal content were determined by plating [5, 9]. Experiments were approved and performed as legally required (Licence 201/2007).

Histology

HE-stained cecum cryosections were scored as described, evaluating submucosal edema, PMN infiltration, goblet cells, and epithelial damage, yielding a total score of 0-13 points [4, 5].

Immunofluorescence Microscopy

PARP1 and PAR were detected by immunofluorescence microscopy as described [19] using cryosections stained with rabbit anti-PARP1 antibody H-250 (Santa Cruz), mouse anti-PAR antibody 10H (kindly provided by Dr. Alexander Bürkle), FITC-conjugated or Cy3-conjugated secondary antibodies (Covance), and DAPI. Images were taken using an Olympus Mx51, NA 1.3 fluorescence microscope.

Western Blot

Cecum tissues were washed in ice-cold PBS, shock frozen in liquid nitrogen, mechanically pulverized and lysed in 50mM Tris-HCl pH 7.5, 400mM NaCl, 25mM NaF, 1% (v/v) Triton X-100. The lysate was used for standard Western blot procedures using anti-PARP1 antibody H-250 (Santa Cruz) or anti-Tubulin (Sigma).

RNA Isolation

The intestinal tract was excised and the cecum was isolated. The cecum was then cut into four slices. Slices 1 and 3 were washed in cold PBS to remove cecum content and

afterwards frozen in liquid nitrogen in 300 µl RLT-buffer (RNeasy Mini Kit, Qiagen) with 1% β-Mercaptoethanol and stored at -80°C till RNA-extraction. Frozen tissue was homogenized in RLT for 3 minutes at 25 Hz using a Tissue Lyser (Qiagen). RNA was processed using the RNeasy Mini Kit (Qiagen) and the RNase-Free DNase Set (Qiagen) according to the manufacturer's instruction. Slices 2 and 4 were cryo-embedded for HE staining.

Quantitative RT-PCR

Total RNA isolated from cecum was reverse-transcribed using the High-Capacity cDNA Reverse Transcription Kit (Applied Biosystems) and used in quantitative real-time (RT) PCR. Reactions were performed with SensimixPlusSYBR (Quantace) and gene-specific primer pairs in Rotor-Gene3000 (Corbett Life Science). Rps12 was used to normalize for differences in cDNA input. RNA from n ≥ 5 individual mice per condition was pooled (except for the 12h time-point, where RNA from only 2 individual mice was pooled). All reactions were performed in triplicates. Primer pairs (5`-3`):

mRps12: GAAGCTGCCAAAGCCTTAGA and AACTGCAACCAACCACCTTC
mGAPDH: GCTACACTGAGGACCAGGTTG and GCCCCTCCTGTTATTATGGGGG
mPARP1: GCAGTCACCCATGTTCGATGG and GCTTCTCTGGATCCACCATC
mCxcl9: GGAGTTCGAGGAACCCTAG and CTTCTTCACATTTGCCGAGTCC
mGbp2: CTTGAAGATGTTGAGAAGGGTGACAACC and GATCAGTTAGCTCCGTCACATAGTGC
mCxcl10: GCACGAACTTAACCACCATCTTCC and CTACCCATTGATACATACTTGATGACAC
mIigp1: GATAGTAGTGTGCTCAATGTTGC and GGTATATTGGGGTGTTTGTATGG
mTgtp: CTCAGGGAGATCCAACTGTCCATCC and CTGTATGGTAGAAGCTCAGCAGTGG
mIgtp: GCTTTGTAAGGCTTCTGAGCAGG and CTGATGAGGCGCTTGAGATAATTTGC
mCd274: CTCCTCGCCTGCAGATAGTTCC and CTTCCTTTTCCCAGTACACCACTAACG
mMpa2l: CTTGGAGAAGCCTACTTCGTCTCT and AAATCTGCCAGCAGACCCTAACCT
mIFNg: CATGGCTGTTTCTGGCTGTTACTG and GTTGCTGATGGCCTGATTGTCTTT

Whole mouse genome arrays

Agilent whole mouse genome arrays 4x44K (Agilent Technologies) were used for the analysis of host gene expression profiles. Total RNA integrity was verified by capillary gel electrophoresis using the 2100 Bioanalyzer (Agilent Technologies). Microarray slides were scanned with an Agilent DNA Microarray scanner and the scans were quantified using the Agilent Feature Extraction software. Background subtraction and dye normalization for each array was performed within the Agilent Feature Extraction software with default settings. The quantified data was subsequently loaded into GeneSpring GX 10 for further analysis. Data from individual mice were grouped according to condition. For the 10h time-point, data from two *S.*tm^{avir}-infected and three *S.*tm-infected wild type mice were compared. For the 6h time-point, data from three *S.*tm-infected wild type and three *S.*tm-

infected knockout animals were compared. P values were obtained by unpaired t-test using GeneSpring GX 10. Gene ontology (GO) and pathway analyses were also performed with GeneSpring GX 10.

Statistical analysis

Pathoscores were analyzed by Mann-Whitney-U-Test, gene expression data was analyzed by unpaired T-Test. Values $p<0.05$ were regarded statistically significant.

Results

Salmonella Typhimurium induces colitis and a pro-inflammatory gene expression response in streptomycin pretreated mice

To study the molecular mechanisms of the host response to enteric salmonellosis, we employed an established mouse model system for *Salmonella*-induced colitis, which relies on the treatment of mice with streptomycin prior to *Salmonella* infection [4]. This system resembles key features of human gut infection by *Salmonella enterica* subspecies 1 serovar Typhimurium [4]. We employed wild type *S.* Typhimurium (*S.*tm) and an isogenic serovar Typhimurium mutant SB161 (*S.*tm^{avir}; SL1344 Δ*inv*G), which lacks an essential subunit of the SP1 type III secretion apparatus and therefore is incapable of actively invading the gut mucosa. The latter served as a negative control strain. Infection of streptomycin-pretreated wild type C57Bl/6J mice with wild type *S.*tm resulted in severe colitis 10h post infection (p.i.), with classical hallmarks of cecal inflammation including pronounced edema in the submucosa, disruption of the crypt architecture, loss of goblet cells, epithelial erosion and infiltration of polymorphonuclear granulocytes (PMNs) (Figure 1A and B). In contrast, and in line with earlier work [4], infection with the mutant *S.*tm^{avir} did not trigger any measurable inflammatory response in the cecum by 10h p.i., although the cecum lumen was heavily colonized.

In order to obtain insights into the *Salmonella*-induced changes at the level of host gene expression, we analyzed gene transcript profiles 10h p.i. by whole mouse genome arrays of cecum from C57Bl/6J mice infected with either wild type *S.*tm. or mutant *S.*tm^{avir}. These microarray studies revealed that 2193 probes corresponding to 1684 genes were up-regulated more than two-fold in *S.*tm infected mice as compared to *S.*tm^{avir} infected control mice (Figure 1C and Supplementary Table 1). 2466 probes corresponding to 1964 genes, on the other hand, were down-regulated more than two-fold in *S.*tm infected mice (Supplementary Table 2). Whereas the up-regulated genes were mainly involved in the immune response as revealed by gene ontology analysis (Figure 1C and Supplementary Table 3), the down-regulated genes had more diverse functions not primarily related to immune system processes (Figure 1C and Supplementary Table 4). To confirm the microarray results by quantitative RT-PCR, we chose two representative up-regulated genes, the neutrophil chemoattractant protein Cxcl9 and the interferon-inducible, antiviral guanylate-binding protein 2 (Gbp2). Both genes were indeed highly induced by *S.*tm as compared to *S.*tm^{avir} (Figure 1D). Thus, the severe inflammation caused by *Salmonella* Typhimurium correlates with pro-inflammatory gene expression 10h p.i..

The protein family nuclear factor-kappaB (NF-κB) comprises an important group of inducible transcription factors involved in pro-inflammatory gene expression and is responsible for the expression of a plethora of cytokines, chemokines, adhesion molecules and inflammatory mediators [20]. A pathway analysis of *S.*tm-induced genes suggested that NF-κB might be involved in the observed expression profile (Supplementary Table 5). Moreover, a search for NF-κB binding sites in the promoter regions of *S.*tm-induced genes identified NF-κB consensus binding sequences in 204 promoters, representing 12 % of all *S.*tm-induced genes. When restricting the search to the most up-regulated genes (with a fold induction of greater than 10), 25 % of the promoters contained NF-κB consensus binding sequences. Together, these analyses suggest an important role of NF-κB for *Salmonella*-induced gene expression.

NF-κB requires several co-activators and co-repressors for the regulation of target gene expression. PARP1 is one of the known transcriptional co-regulators of NF-κB and PARP1 knockout mice are protected from tissue injury in various inflammation-related disease models [21]. This led us to hypothesize that PARP1 might also be important for *S.*tm-induced colitis.

PARP1 is expressed in the proliferative zone of cecum crypts

We first analyzed whether PARP1 was expressed in the mouse cecum, where *S.*tm-induced inflammation was most severe. PARP1 mRNA was detected in the cecum of wild type but not isogenic PARP1 knockout mice as revealed by quantitative RT-PCR (Figure 2A). PARP1 was also detected at the protein level by Western blot analysis of cecal extracts (Figure 2B). Immunofluorescence staining confirmed PARP1 expression in the cecum, predominantly in the proliferative zone of the crypts, whereas PARP1 was absent in knockout animals (Figure 2C).

After having confirmed that PARP1 was expressed in the mouse cecum, we infected wild type and PARP1 knockout mice with either wild type *S.*tm or with the mutant strain *S.*tm^{avir}. Neither PARP1 mRNA nor protein levels changed in the cecum of wild type animals after *S.*tm infection (Supplementary Figure 1A and data not shown). Although we were able to detect poly(ADP-ribose), the reaction product of PARP1, in H_2O_2-treated transformed gastric epithelial cells (Supplementary Figure 1B), we were not able to detect poly(ADP-ribose) in cecum cryosections at 6h to 12h after *Salmonella* infection (Supplementary Figure 1C). Thus, neither PARP1 expression nor its catalytic activity is significantly altered after *S.*tm infection.

PARP1 accelerates *Salmonella*-induced inflammation

Next, we analyzed *S.*tm-induced pathology in wild type and PARP1 knockout mice, which had been sacrificed 6h, 8h, 10h, or 12h p.i.. Whereas at 6h p.i., neither the wild type nor the PARP1 knockout animals showed signs of mucosal pathology when infected with *S.*tm, at 8h p.i. the wild type mice showed considerable signs of inflammation (Figure 3A, black symbols). Strikingly, PARP1 knockout animals had significantly lower pathoscores at this time-point. Submucosal edema, partly disrupted crypts, beginning erosion of the epithelium and loss of goblet cells were observed in wild type mice but were significantly less pronounced in knockout mice 8h p.i. (Figure 3B). At 10h p.i., first signs of inflammation were also observed in PARP1 knockout mice (Figure 3A). The difference between wild type and PARP1 knockout animals was eventually lost 12h p.i. (Figure 3A). Thus, we observed a significant delay in the host response to *Salmonella* Typhimurium infection in PARP1 knockout mice as compared to wild type control animals. This delay was not due to differences in cecum colonization, because wild type and isogenic PARP1 knockout mice had comparable bacteria loads (Figure 3C). Neither wild type nor PARP1 knockout mice reacted to the *S.*tm^{avir} control strain with an inflammatory response (Figure 3D), but *S.*tm^{avir} was able to colonize the cecum lumen of wild type and knockout mice as efficiently as *S.*tm (Figure 3E). We therefore conclude that PARP1 knockout animals have a specific defect in the early pro-inflammatory response. Accordingly, PARP1 is a novel host factor, which accelerates inflammation caused by *Salmonella* Typhimurium infection.

To assess if the accelerated inflammation observed in PARP1 proficient mice was a consequence of increased production of cytokines, we analyzed pro-inflammatory gene expression in cecum preparations from wild type and PARP1 knockout mice by quantitative RT-PCR. Remarkably, the expression of Cxcl9 and Gbp2 was robustly increased at 6 and 8h p.i. in ceca from wild type mice but not from PARP1 knockout animals (Figure 3F). In line with the histopathologic data, both genes were almost as efficiently transcribed in knockout animals as compared to wild type controls at the later time-points 10 and 12h p.i. (Figure 3F). Together, these data suggest an impaired immediate gene expression response to *S.* Typhimurium infection in PARP1 knockout mice.

PARP1 is required for the efficient expression of a subset of *Salmonella*-induced genes

No signs of inflammation could be observed by histopathologic analysis of the cecum of wild type or PARP1 knockout animals at the earliest time-point we investigated (6h p.i.). The pro-inflammatory gene expression at this time-point therefore most likely

represents an early, primary host response of the mucosal epithelial cell layer to the invading pathogen, which precedes and is still independent of the infiltration of immune cells. We thus decided to focus on this early time-point to study the mechanism, which might be responsible for the delayed onset of colitis in PARP1 knockout mice. 6h p.i., the basal levels of pro-inflammatory genes in control-infected animals were normal in mice lacking PARP1 (Figure 3F). Moreover, so-called housekeeping genes like GAPDH were also expressed normally in PARP1 knockout mice (Supplementary Figure 2). However, the induction of Cxcl9 and Gbp2 after *S.*tm infection was clearly reduced in knockout animals already at this early time-point, strongly suggesting that the immediate gene expression response of the host tissue was impaired. To determine the extend of pro-inflammatory genes, which depend on PARP1 for efficient expression after *Salmonella* infection, whole genome arrays from wild type and PARP1 knockout mice infected with *S.*tm for 6h were performed. We were primarily interested in genes, which were induced by *S.*tm (Figure 1C) and showed reduced expression in PARP1 knockout animals. 65 genes fulfilled these criteria, as they were *Salmonella*-induced and showed at least a 1.5-fold reduction in *S.*tm-infected PARP1 knockout mice as compared to *S.*tm-infected wild type animals (Figure 4A and Supplementary Table 6). In contrast, only 4 genes were repressed by *Salmonella* infection and lower expressed in PARP1 knockout mice as compared to wild type controls. Thus, of the 1684 genes, which showed an up-regulation 10h after *Salmonella* infection, 65 genes, or 3.86%, were dependent on PARP1 at 6h p.i. (Figure 4B). This suggests, that PARP1 acts as transcriptional co-activator for a defined subset of *Salmonella*-induced genes. A gene ontology analysis revealed that many of these PARP1-dependent genes are involved in the immune response (Figure 4C and Supplementary Table 7) and, according to a pathway analysis, are related to IFNγ signaling (Figure 4D and Supplementary Table 8). In detail, besides Cxcl9 and Gbp2, these genes include interferon-inducible protein 10 (IP-10/Cxcl10), interferon-inducible GTPase 1 (Iigp1), T-cell specific GTPase (Tgtp), IFNγ-induced GTPase (Igtp), CD274 antigen, macrophage activation 2 like (Mpa2l), and others (Supplementary Table 6). When we directly tested IFNγ transcript levels after *S.*tm infection in wild type and PARP1 knockout animals by quantitative RT-PCR, we found a slight but not significant difference in IFNγ gene expression, suggesting that IFNγ levels per se may not be the primary cause for the impaired expression of some IFNγ-related genes in PARP1 knockout mice (Figure 5A). We confirmed several PARP1 dependent genes 6h p.i. by quantitative RT-PCR and also extended the analysis to the 8h time-point. In confirmation of the microarray results, many tested genes showed a clear dependence on PARP1 for efficient transcription 6h after infection (Figure 5B), and in line with the

pathological differences between wild type and PARP1 knockout mice 8h p.i., all genes were greatly reduced in knockout animals at this later time-point (Figure 5C). A summary of all analyzed genes is provided in Table 1. Together, our data provide evidence that PARP1 is required for the efficient expression of a subset of *Salmonella*-induced genes *in vivo*. Efficient PARP1-dependent expression of these genes likely contributes to the activation of resident immune cells and/or recruitment of inflammatory cells to trigger an inflammatory response. Thus, our data identify PARP1 as a host factor controlling the early phase of *Salmonella*-induced inflammation.

Discussion

Here we show that the nuclear protein PARP1 is linked to salmonellosis. In an *in vivo* mouse model system for *Salmonella*-induced colitis, we demonstrate that PARP1 is a novel host factor, which accelerates inflammation after *Salmonella* infection. PARP1 is predominantly expressed in the proliferative zone of the crypts and is required for the efficient early expression of several *Salmonella*-induced pro-inflammatory genes. Reduced early pro-inflammatory gene expression in mice lacking PARP1 correlates with and may be causally responsible for delayed infiltration of immune cells and consequently delayed onset of inflammation. Together, our findings reveal that PARP1 is required for an efficient early innate immune response against *Salmonella* infection.

While we observed a significantly reduced pro-inflammatory response in PARP1 knockout mice at early time-points after *Salmonella* infection, PARP1 knockout animals also developed severe colitis later on. Therefore, the innate immune system does not completely rely on PARP1-dependent mechanisms, but instead can compensate for the lack of PARP1 at later time-points. We would speculate that the immune system senses the dampened response to *Salmonella* infection in the absence of PARP1 and then triggers delayed compensatory, PARP1-independent mechanisms to achieve an adequate host response.

The function of PARP1 as transcriptional co-regulator is now well established [21]. Besides NF-κB, also other transcription factors, including nuclear factor of activated T cells (NFAT), activator protein-1 (AP-1), forkhead box O transcription factor 1 (FOXO1), and nuclear respiratory factor 1 (NRF-1), were described to depend on PARP1 for efficient target gene expression [22-25]. PARP1-dependent genes typically are not completely silenced in cells lacking PARP1, but instead show a 30-70% reduced expression (own unpublished observation). Moreover, PARP1 seems to be more important for induced gene expression after cell stimulation as compared to basal gene expression under non-stimulated conditions. For example, in a study by Saenz et al., only 93 genes (0.66%) were found differentially expressed under normal conditions in wild type and PARP1 knockout T cells, while 203 genes (1.44%) were found differentially expressed after anti-CD3/anti-CD28 stimulation [26].

IFNγ is the sole type II interferon and an important mediator of immunity and inflammation [27]. Consequently, IFNγ is found among the cytokines whose expression is most prominently induced during *Salmonella* infection [28]. We observed increased IFNγ expression after *Salmonella* infection by both microarray analysis and quantitative RT-

PCR. Production of IFNγ in the early phase of intestinal inflammation contributes to antimicrobial responses in the intestinal mucosa [29] and may be required to amplify initial responses generated by bacterial host cell interaction [28]. Moreover, IFNγ priming increases TLR expression, promotes NF-κB activation, and induces transcription factors, which are essential for expression of certain TLR-responsive genes [27]. Our data revealed that a subset of IFNγ response genes requires PARP1 for early efficient expression. These genes may be driven by NF-κB alone or may require multiple transcription factors, possibly including NF-κB, STAT-1, and IRFs, at their promoter sites for efficient expression after *Salmonella* infection. Many of the identified genes, including Cxcl9, Cxcl10, Iigp1 and Cd274, are reported NF-κB target genes or contain κB-sites (http://people.bu.edu/gilmore/nf-kb/index.html and data not shown). In line with the notion that NF-κB may be involved in the regulation of PARP1-dependent IFNγ response genes, STAT-1, the primary transcription factor down-stream of IFNγ signaling, is often necessary but not sufficient for transcription of target genes [30, 31]. In fact, the ability of STAT-1 to activate gene expression often depends on the presence of other transcription factors binding to the promoter element [32]. Thus, in many cases, synergistic gene induction by a combination of pro-inflammatory stimuli may be a result of the combined presence of STAT-1 and NF-κB at the promoter elements of responsive genes.

In conclusion, our data directly link PARP1 to the pathogenesis of *Salmonella*-induced inflammation and provide a plausible mechanistic explanation how PARP1 accelerates colitis. These findings increase our understanding on how the nuclear multi-functional protein PARP1 is involved in pro-inflammatory gene expression and how it is implicated in pro-inflammatory diseases. Our results also shed light on the regulation of the early host response after *Salmonella* infection and may prove beneficial for the design of new therapeutic strategies to combat salmonellosis.

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Figure Legends

Figure 1: *Salmonella*-induced inflammation 10h post infection in wild type mice. (A) Signs of inflammation and cecum colonization were analyzed in wild type C57Bl/6J mice infected with *Salmonella* strain *S.*tm^{avir} or *S.*tm. Cecal pathology was scored and bacteria loads were determined as described in the Methods section. n=5 animals in each group were analyzed. Medians are indicated. (B) Representative HE-stained cecum cryosections. L, lumen; g, goblet cell; SM, submucosa; e, edema; er, erosion of the epithelial layer. Bar = 100μm. (C) Whole mouse genome array data from wild type mice infected with either *S.*tm^{avir} or *S.*tm for 10h. Total numbers of up-regulated genes (>2-fold, $p<0.05$) and down-regulated genes (>2-fold, $p<0.05$) are indicated. Highest scoring GO-terms for up-regulated genes (top) and down-regulated genes (bottom) are listed. (D) Quantitative RT-PCR confirmation for the indicated genes. Values obtained for *S.*tm^{avir}-infected mice were set 1. Reactions were performed in triplicates, mean values ± SD are shown.

Figure 2: PARP1 is expressed in the proliferative crypts of the cecum. (A) Quantitative RT-PCR analysis of PARP1 mRNA levels in mouse cecum from wild type (wt) and PARP1 knockout (ko) mice. Wild type mRNA expression levels were set 1. Reactions were performed in triplicates, mean values ± SD are shown. (B) Western blot analysis of PARP1 protein expression in mouse cecum from wild type and PARP1 knockout mice. (C) PARP1 immunofluorescence in cecum cryosections from wild type and PARP1 knockout mice. L, lumen. Bar = 100μm.

Figure 3: PARP1 accelerates *Salmonella*-induced inflammation. (A) Histopathologic analysis of wild type (wt) and isogenic PARP1 knockout (ko) mice after 6h (n=8 per group), 8h (n=15 for wt, n=12 for ko), 10h (n=5 per group), and 12h (n=2 per group) after *S.*tm infection. Medians are indicated. p-values were obtained by Mann-Whitney-U-Test. (B) Representative HE-stained cecum cryosections 8h after *S.*tm infection. L, lumen; g, goblet cell; SM, submucosa; er, erosion of the epithelial layer. Bar = 100μm. (C) Cecum colonization of wild type and PARP1 knockout mice after 6h (n=8 per group), 8h (n=15 for wt, n=12 for ko), 10h (n=5 per group), and 12h (n=2 per group) after *S.*tm infection. Medians are indicated. (D) Histopathologic analysis of wild type and PARP1 knockout mice after 6h (n=8 per group), 8h (n=15 for wt, n=12 for ko), 10h (n=5 per group), and 12h (n=2 per group) after *S.*tm^{avir} infection. Medians are indicated. (E) Cecum colonization of wild type and PARP1 knockout mice after 6h (n=8 per group), 8h (n=15 for wt, n=12 for

ko), 10h (n=5 per group), and 12h (n=2 per group) after *S.*tm^{avir} infection. Medians are indicated. (F) Quantitative RT-PCR analysis of the indicated genes at the indicated time-points. Values obtained for *S.*tm^{avir}-infected wild type mice were set 1. Reactions were performed in triplicates, mean values ± SD are shown.

Figure 4: A subset of *Salmonella*-induced genes requires PARP1 for efficient expression. (A) Comparison of whole mouse genome microarray data. *Salmonella*-induced and *Salmonella*-repressed genes (*S.*tm over *S.*tm^{avir}, 10h p.i.) are shown in grey. *Salmonella*-induced and *Salmonella*-repressed genes (*S.*tm over *S.*tm^{avir}, 10h p.i.), whose expression was lower in *S.*tm-infected PARP1 knockout mice as compared to *S.*tm-infected wild type mice (6h p.i.), are shown in black and are indicated by numbers. (B) Relative number of PARP1-dependent, *Salmonella*-induced genes. (C) Pathway analysis of PARP1-dependent, *Salmonella*-induced genes. (D) Gene ontology (GO) analysis of PARP1-dependent, *Salmonella*-induced genes.

Figure 5: Impaired gene induction in PARP knockout mice 6h p.i. persists and is more dramatic 8h p.i.. (A) Quantitative RT-PCR analysis of IFNγ 6h p.i.. Fold inductions over *S.*tm^{avir}-infected controls are presented. Reactions were performed in triplicates, mean values ± SD are shown. (B) Quantitative RT-PCR analysis of the indicated genes 6h p.i.. Fold inductions over *S.*tm^{avir}-infected controls are presented. Reactions were performed in triplicates, mean values ± SD are shown. (B) Quantitative RT-PCR analysis of the indicated genes 8h p.i.. Fold inductions over *S.*tm^{avir}-infected controls are presented. Reactions were performed in triplicates, mean values ± SD are shown.

Table Legends

Table 1: Summary of gene expression analysis for selected genes and time-points after *Salmonella* infection. Gene expression data obtained by whole mouse genome arrays and quantitative RT-PCR are presented.

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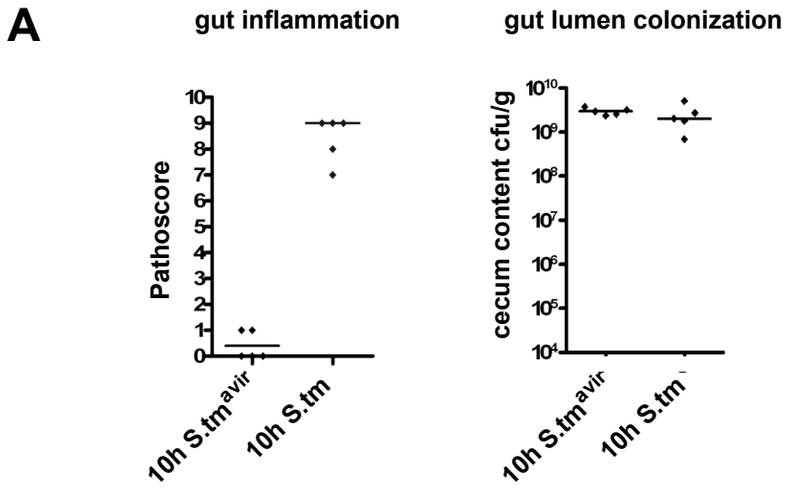
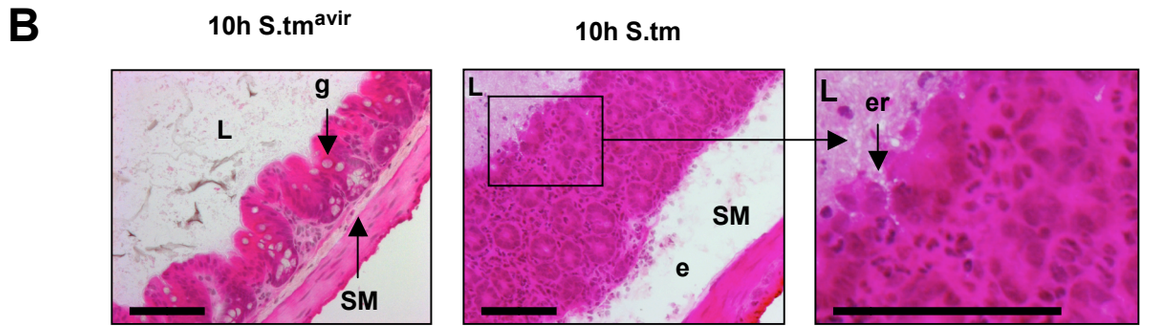
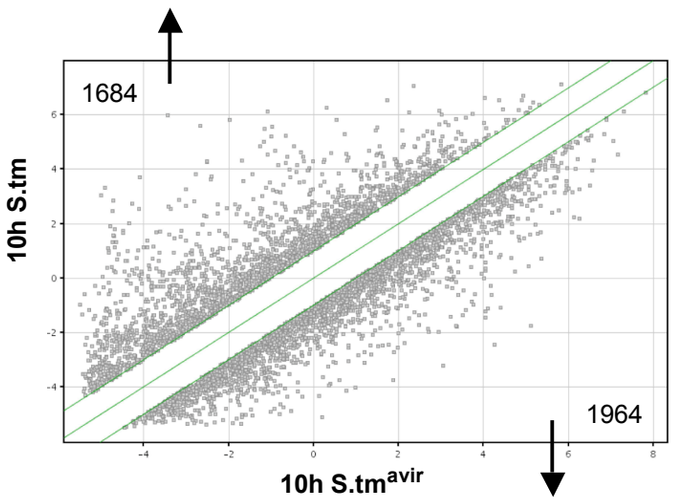




C

GO Term	p value	corrected p value
immune system process	1.29E-32	3.33E-27
response to external stimulus	4.49E-31	5.79E-26
response to wounding	4.20E-30	3.61E-25
immune response	6.92E-28	4.46E-23
inflammatory response	2.27E-27	1.17E-22
defense response	4.68E-26	2.01E-21



GO Term	p value	corrected p value
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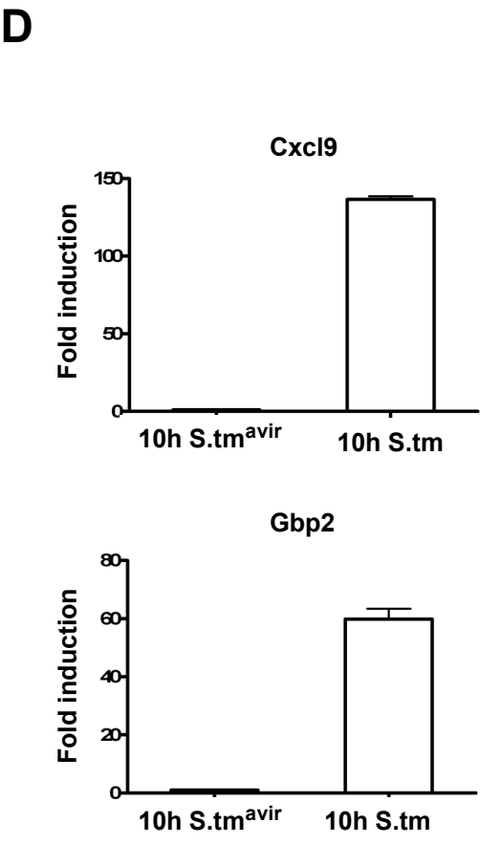
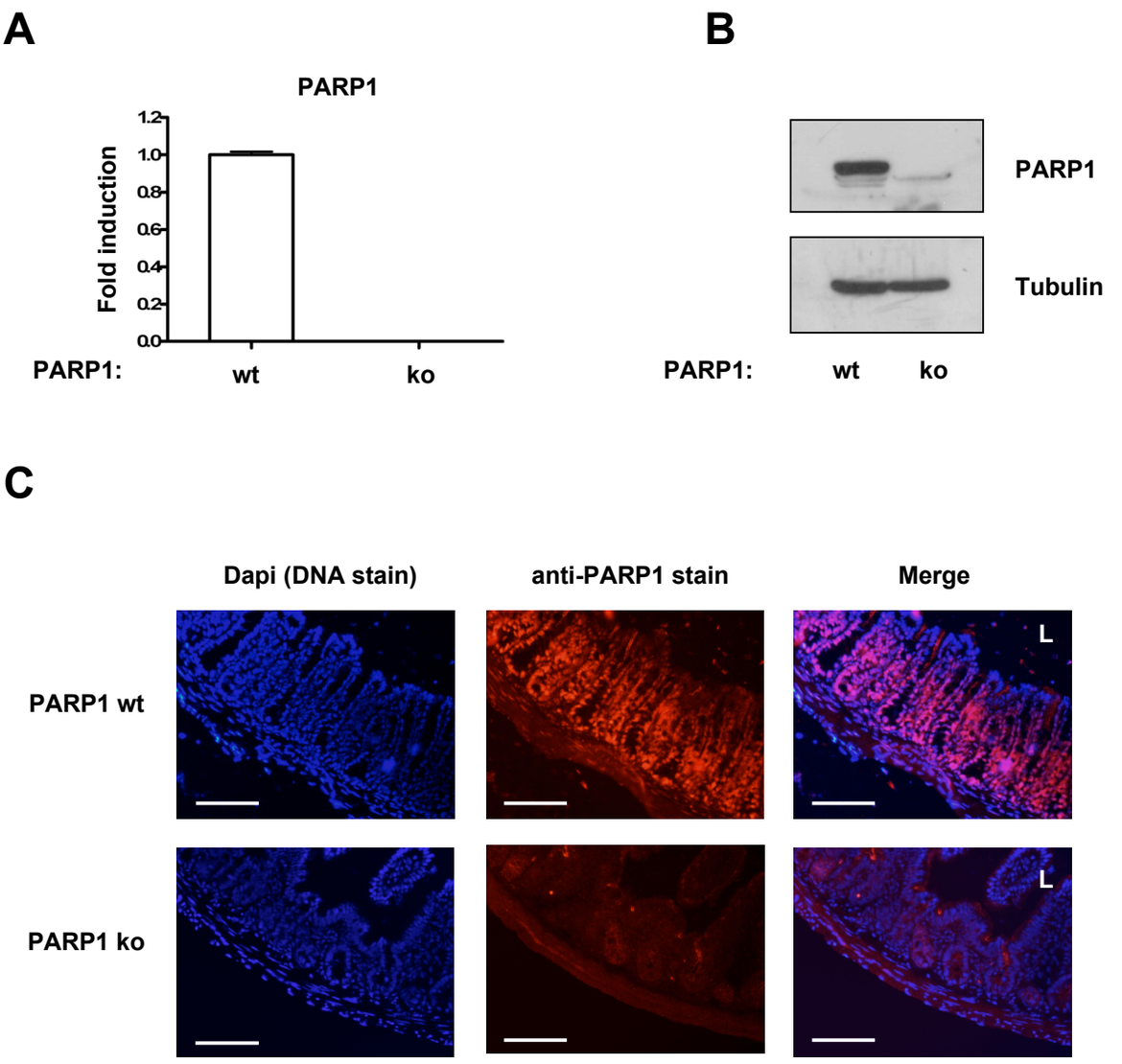
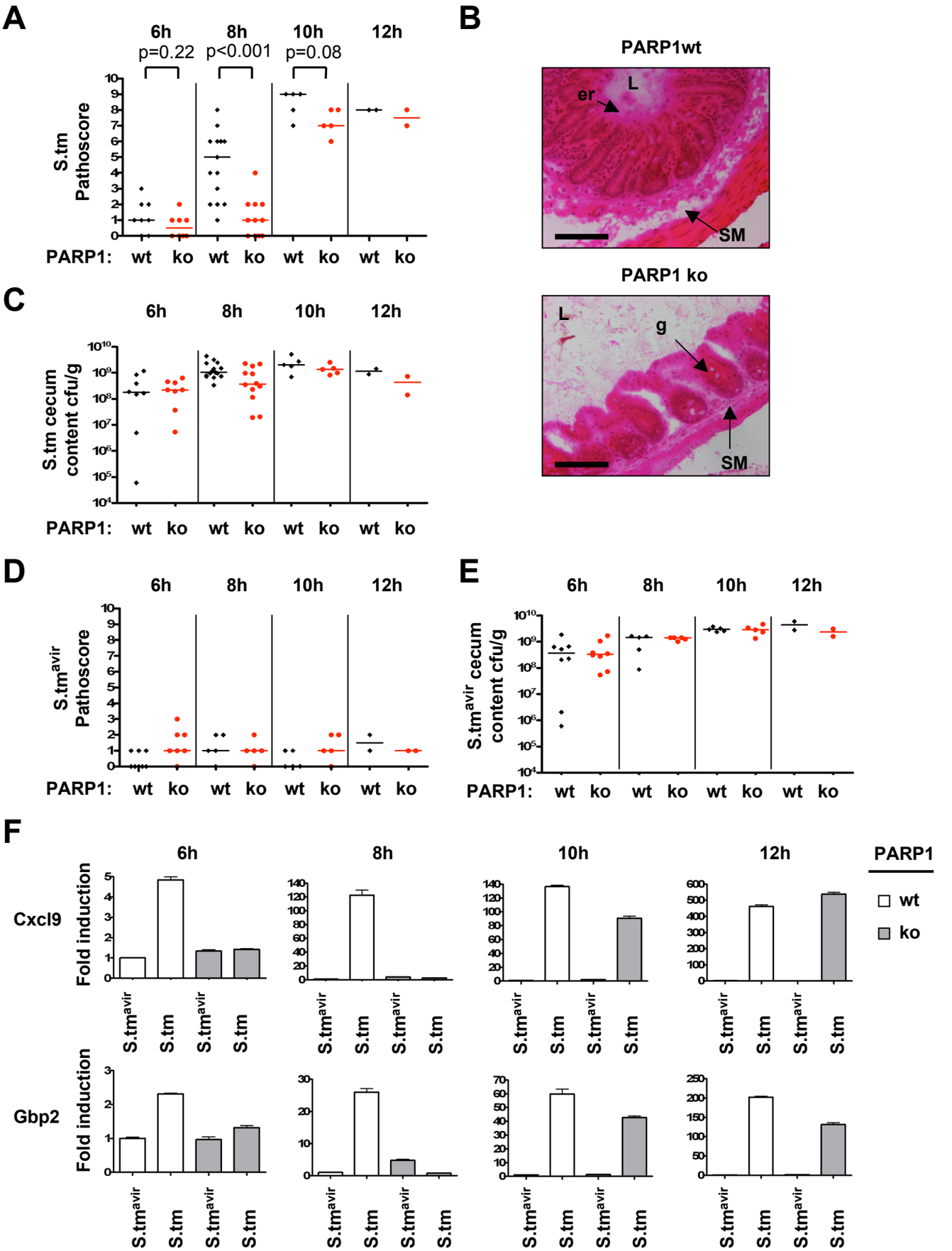
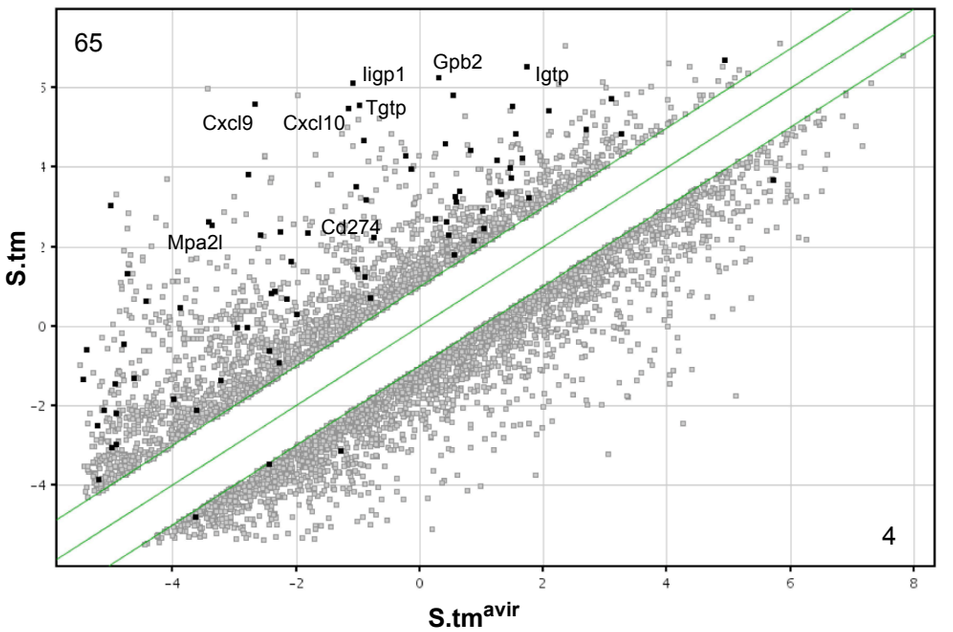
lipid metabolic process	6.06E-25	7.81E-20
cellular lipid metabolic process	1.15E-24	9.87E-20



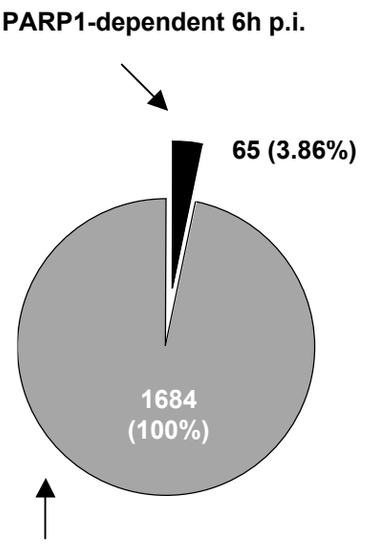

A
PARP1
Fold induction
1.2
1.0
0.8
0.6
0.4
0.2
0.0
PARP1:
wt
ko
B
PARP1
Tubulin
PARP1:
wt
ko
C
Dapi (DNA stain)
anti-PARP1 stain
Merge
PARP1 wt
PARP1 ko
L
L


A
6h
8h
10h
12h
p=0.22
p<0.001
p=0.08
S.tm Pathoscore
PARP1: wt ko wt ko wt ko wt ko
B
PARP1wt
er
L
SM
PARP1 ko
L
g
SM
C
6h
8h
10h
12h
S.tm cecum content cfu/g
PARP1: wt ko wt ko wt ko wt ko
D
6h
8h
10h
12h
S.tmavir Pathoscore
PARP1: wt ko wt ko wt ko wt ko
E
6h
8h
10h
12h
S.tmavir cecum content cfu/g
PARP1: wt ko wt ko wt ko wt ko
F
6h
8h
10h
12h
PARP1
wt
ko
Cxcl9
Fold induction
Gbp2
Fold induction
S.tmavir
S.tm

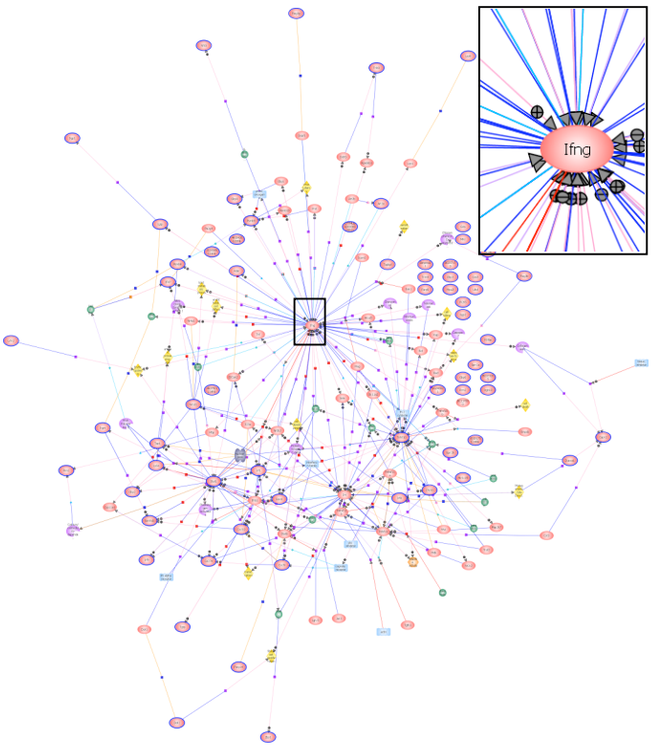
A



B

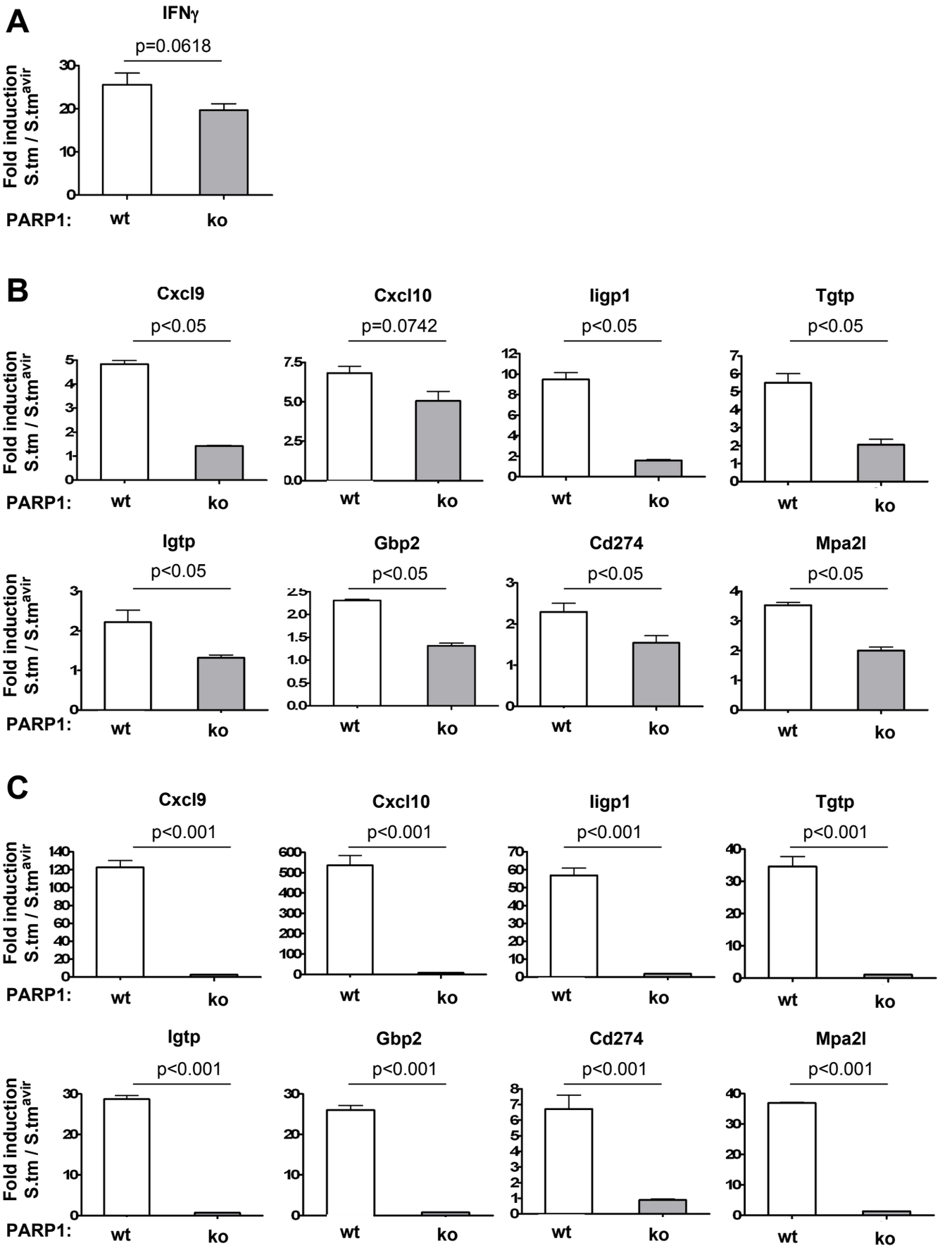


D



C

GO Term	p-value	corrected p-value
immune response	1.75E-16	4.52E-11
immune system process	5.17E-14	6.67E-09
GTPase activity	9.80E-11	8.42E-06

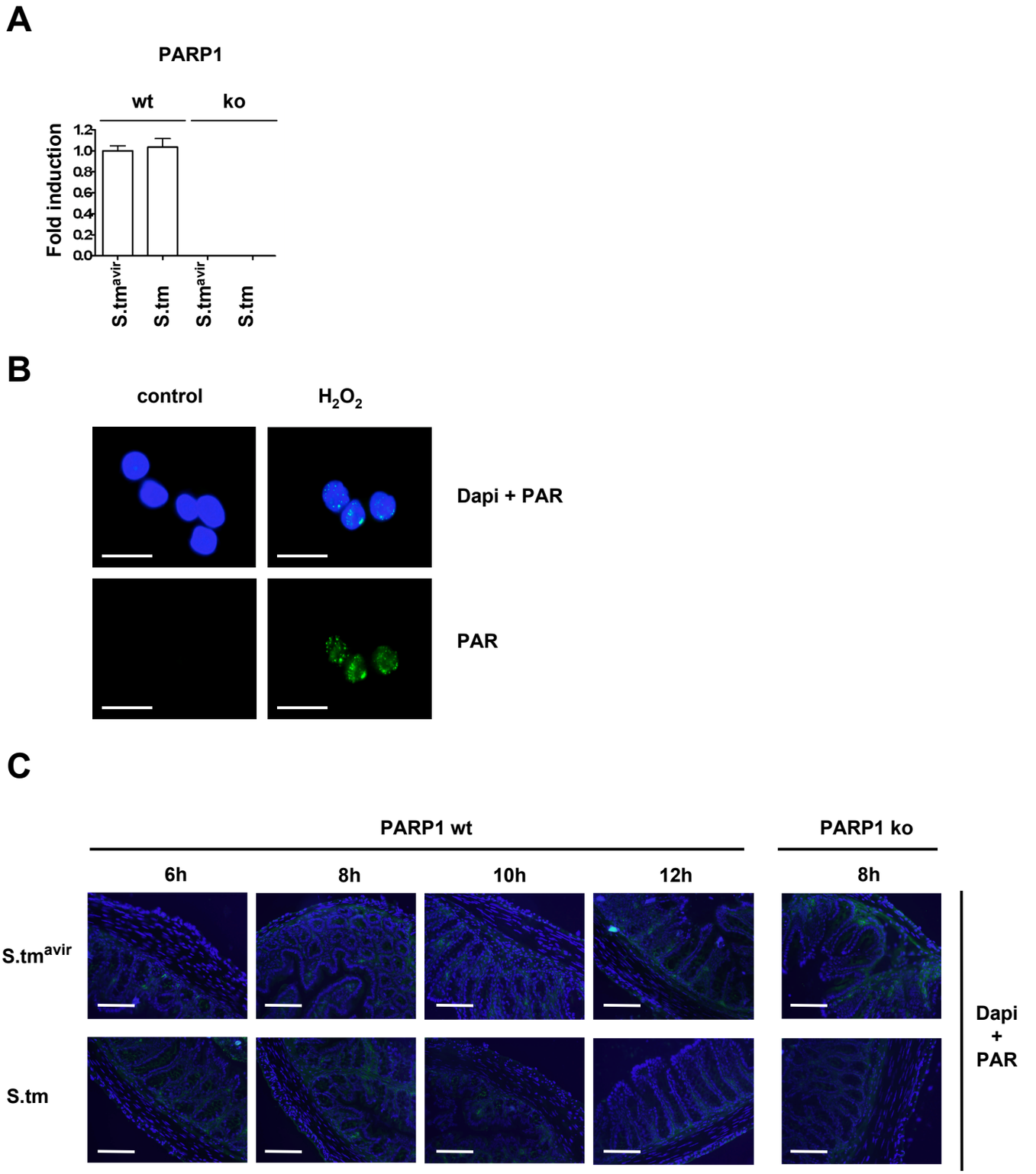

A
IFNγ
p=0.0618
Fold induction S.tm / S.tm^avir
PARP1: wt ko
B
Cxcl9
p<0.05
Cxcl10
p=0.0742
Iigp1
p<0.05
Tgtp
p<0.05
Igtp
p<0.05
Gbp2
p<0.05
Cd274
p<0.05
Mpa2l
p<0.05
C
Cxcl9
p<0.001
Cxcl10
p<0.001
Iigp1
p<0.001
Tgtp
p<0.001
Igtp
p<0.001
Gbp2
p<0.001
Cd274
p<0.001
Mpa2l
p<0.001

		Salmonella-induced (S.tm/S.tm_avir)			**PARP1-dependent (ko/wt)**		
		array 10h p.i.		qRT-PCR 6h p.i.	array 6h p.i.	qRT-PCR 6h p.i.	qRT-PCR 8h p.i
No	**GeneSymbol**	**Fold induction**	**Rank**	**Fold induction**	**Fold reduction**	**Fold reduction**	**Fold reduction**
1	Cxcl9	309.33	2	4.84	2.33	3.43	45.23
2	Iigp1	149.09	8	9.48	3.46	5.23	35.70
3	Cxcl10	98.23	15	6.83	1.72	1.35	81.95
4	Tgtp	93.33	17	5.50	1.56	2.69	33.54
5	Gbp2	61.07	37	2.31	2.91	1.76	34.16
6	Mpa2l	59.90	38	3.53	2.32	1.77	29.87
7	Socs1	47.60	46	1.54	2.10	0.92	19.11
8	Igtp	27.75	80	2.22	3.52	1.68	42.80
9	Gbp6	24.85	87	ND	2.00	ND	ND
10	Gbp3	22.79	100	ND	2.37	ND	ND
11	Iigp2	16.52	139	ND	2.61	ND	ND
12	Cd274	7.93	295	2.30	2.48	1.49	7.56
13	Stat1	7.56	308	1.63	1.73	1.10	11.71
14	Il18bp	7.20	321	ND	2.23	ND	ND

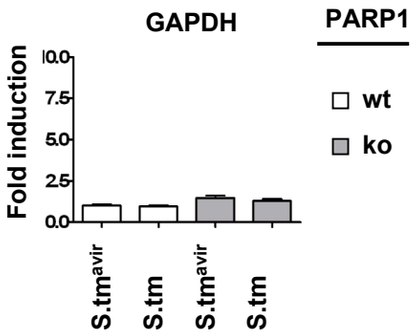
Supplementary Figure Legends

Supplementary Figure 1: (A) Quantitative RT-PCR for PARP1 at 6h p.i.. Values obtained for *S.*tm^{avir}-infected mice were set 1. Reactions were performed in triplicates, mean values ± SD are shown. (B) Poly(ADP-ribose) (PAR) immunofluorescence in AGS gastric epithelial cells after treatment for 10 minutes with 10mM H_2O_2. Bar = 10μm. (C) PAR immunofluorescence in cecum cryosections from wild type and PARP1 knockout mice infected with *S.*tm^{avir} or *S.*tm for the indicated time-periods. Bar = 100μm.

Supplementary Figure 2: Quantitative RT-PCR for GAPDH at 6h p.i.. Values obtained for *S.*tm^{avir}-infected mice were set 1. Reactions were performed in triplicates, mean values ± SD are shown.


A
PARP1
wt
ko
Fold induction
1.2
1.0
0.8
0.6
0.4
0.2
0.0
S.tm^avir
S.tm
S.tm^avir
S.tm
B
control
H2O2
Dapi + PAR
PAR
C
PARP1 wt
PARP1 ko
6h
8h
10h
12h
8h
S.tm^avir
S.tm
Dapi
+
PAR

Altmeyer et al. Suppl. Fig.2



2.4 Unpublished data:

2.4.1 Biochemical characterization of PARP family members

2.4.1.1 Recombinant PARP1 preparation

In vitro PARylation analyses were generally performed with recombinant purified full-length human PARP1 containing a carboxyl-terminal His-tag (hPARP1-His) [150]. We noted that expression and one-step purification via Ni-beads of insect cell-expressed hPARP1-His resulted in a second protein band at about 80kDa (Figure 4A). This band was not present when PARP1 contained an additional Myc- or HA-tag at the amino-terminus (Figure 4A and data not shown). The band at about 80kDa in hPARP1-His preparations was readily detected by an antibody against the catalytic domain of PARP1 (Figure 4B), suggesting that this band corresponds to a carboxyl-terminal degradation product of PARP1. The degradation product did not alter auto-modification of PARP1 (Figure 4C) or PARylation activity (Figure 4D). Together, we conclude that an additional amino-terminal tag prevents PARP1 cleavage during expression and purification using the baculovirus system. The degradation product present in hPARP1-His preparations does not influence PARP1 enzymatic activities.

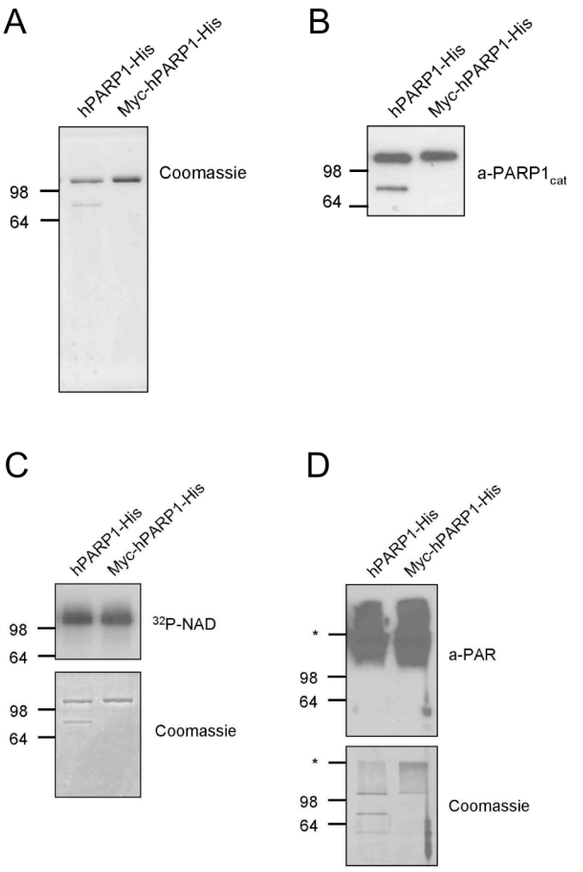


Figure 4: A degradation product of recombinant purified hPARP1-His has no influence on enzymatic activity. (A) hPARP1 was expressed with either only a carboxyl-terminal His-tag or with an amino-terminal Myc-tag and a carboxyl-terminal His-tag in Sf21 insect cells and purified via nickel beads as described [150]. 1 µg of each purified protein was analyzed by SDS-PAGE followed by Coomassie staining. (B) 10 ng of each purified protein was analyzed by SDS-PAGE followed by Western blot. The second protein band at around 80kDa in the hPARP1-His preparation is detected by an antibody directed against the catalytic domain of PARP1 and most likely represents a PARP1 degradation product. (C) The PARP1 degradation product has no influence on auto-modification of PARP1 or (D) on PAR formation. Reactions were performed as described previously [150].

2.4.1.2 Characterization of *in vitro* synthesized PAR

Analytical anion exchange HPLC can be used to characterize PARP1-generated heterogenous mixtures of PAR, which contain polymers of different chain lengths and branching frequency [61]. We used a TSK-GEL anion exchange column to separate *in vitro* generated PAR via HPLC. With a multi-step NaCl gradient it was possible to obtain a resolution of one ADP-ribose moiety and to separate PAR chains differing in only one unit (Figure 5A). PAR ranging from 1-2 ADP-ribose units up to 40-45 units were readily detected with 7.14 nmol PAR input (Figure 5A). The detection limit of this method probably is in the range of 4-5 nmol PAR input (Figure 5A and 5B). Thus, anion exchange chromatography is a suitable method to characterize *in vitro* generated PAR but most likely is not sensitive enough to analyze *in vivo* synthesized PAR.

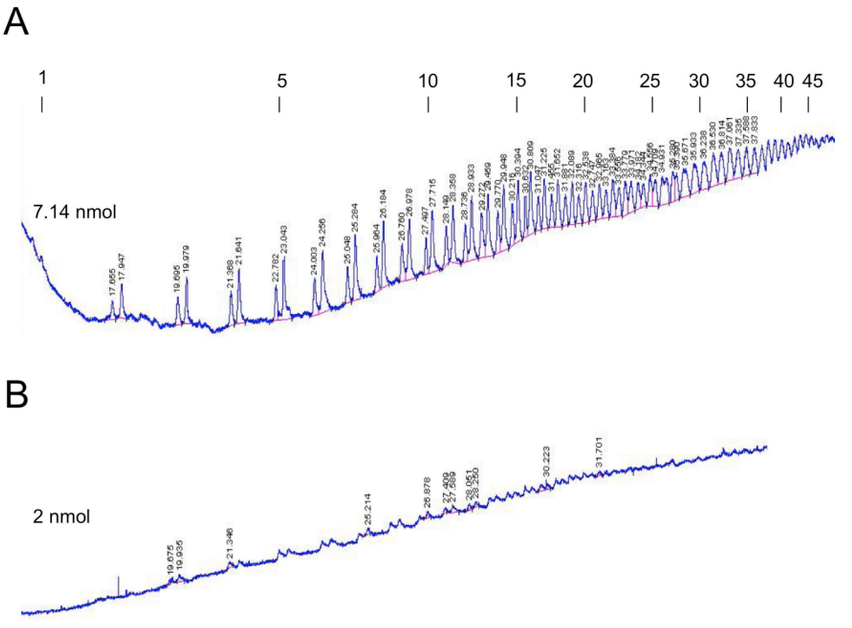


Figure 5: A purified heterogenous mixture of PAR chains can be separated according to chain length by TSK-GEL anion exchange HPLC. (A) PAR was generated *in vitro*, purified and separated via a TSK-GEL anion exchange column (DEAE-NPR, 2.5µm particle size, length x I.D. 3.5cm x 4.6mm, from Tosoh Bioscience) using a multi-step NaCl gradient as described in [61]. 7.14 nmol PAR was used as input. PAR chains contained up to 45 units and could be separated with a resolution of one ADP-ribose moiety. (B) As in (A) with only 2 nmol PAR input.

2.4.1.3 Characterization of PARP3 enzymatic activity

Using standardized reaction conditions to compare the catalytic activities of PARP1, PARP2 and PARP3, we did not observe auto-modification or PARylation by human PARP3 [150]. An increase in substrate concentration by a factor of 100 from 100 nM to 10 μM together with an increase in incubation time by a factor of 90 from 10 seconds to 15 minutes resulted in detectable auto-modification of PARP3 (Figure 6A). The addition of 250 μM DTT resulted in a slight reduction of PARP3 auto-modification activity (Figure 6B, compare lanes 6 and 7). Together, human PARP3 shows auto-modification activity under certain well-defined experimental conditions. No indication for PARylation activity by PARP3 (e.g. no shift in coomassie stained gels, no detectably PAR formation in Western blots using anti-PAR antibodies, no TCA-precipitable PAR formation) was observed. Thus, it remains to be experimentally shown whether PARP3 is truly a PAR polymerase or merely a mono-ADP-ribosyl transferase.

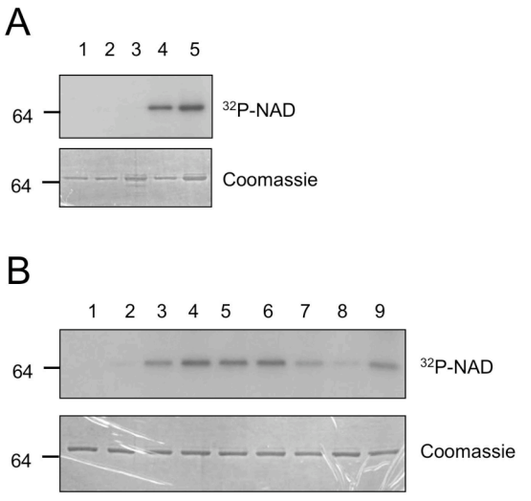


Figure 3: hPARP3 has auto-modification activity. (A) 1: 0.5μg hPARP3, 100nM ^{32}P-NAD, 5pmol EcoRI, 10 sec, PARP reaction buffer [150]; 2: 0.5μg hPARP3, 100nM ^{32}P-NAD, 5pmol EcoRI, 15 min, PARP reaction buffer [150]; 3: 1μg hPARP3, 10μM ^{32}P-NAD, 10 sec, 100mM Tris-HCl pH 8.0; 4: 1μg hPARP3, 10μM ^{32}P-NAD, 15 min, 100mM Tris-HCl pH 8.0; 5: 3.5μg hPARP3, 10μM ^{32}P-NAD, 15 min, 100mM Tris-HCl pH 8.0. (B) 1: 0.5μg hPARP3, 100nM ^{32}P-NAD, 5pmol EcoRI, 10 sec, PARP reaction buffer [150]; 2: 0.5μg hPARP3, 100nM ^{32}P-NAD, 15 min, PARP reaction buffer [150]; 3: 0.5μg hPARP3, 10μM ^{32}P-NAD, 15 min, PARP reaction buffer [150]; 4: 0.5μg hPARP3, 10μM ^{32}P-NAD, 15 min, 100mM Tris-HCl pH 8.0; 5: 0.5μg hPARP3, 10μM ^{32}P-NAD, 15 min, 100mM Tris-HCl pH 8.0, P/B/L; 6: 0.5μg hPARP3, 10μM ^{32}P-NAD, 15 min, 100mM Tris-HCl pH 8.0, P/B/L, 4mM $MgCl_2$; 7: 0.5μg hPARP3, 10μM ^{32}P-NAD, 15 min, 100mM Tris-HCl pH 8.0, P/B/L, 4mM $MgCl_2$, 250μM DTT; 8: 0.5μg hPARP3, 100nM ^{32}P-NAD, 15 min, 100mM Tris-HCl pH 8.0; 9: 0.5μg hPARP3, 10μM ^{32}P-NAD, 15 min, 100mM Tris-HCl pH 8.0, 100mM NaCl. P/B/L: 1μg/ml pepstatin, 1μg/ml bestatin, 1μg/ml leupeptin.

2.4.1.4 Mechanism of PARP1 activation and identification of PAR acceptor amino acids

In order to test whether PARP1 was only activated by double strand break mimicking DNA or also by double stranded blunt ended RNA, we used protein chimera PARP1-2 in *in vitro* PARylation assays [150]. This chimera contains the amino-terminus of PARP1, which harbors three zinc-binding motifs and binds to DNA single and double strand breaks, and the carboxyl-terminus of PARP2. PARP1-2 is activated by a double strand break mimicking DNA oligonucleotide when etheno-NAD^+ is used as substrate (Figure 7A). When we tested a double strand break mimicking RNA oligonucleotide of the same sequence, we did not observe PARP1-2 activation, suggesting that the DNA binding domain of PARP1 does not recognize blunt ended double stranded RNA (Figure 7A).

By using a triple lysine-to-arginine mutant of PARP1 for *in vitro* auto-modification reactions, we had identified the lysine residues K498, K521 and K524 in the auto-modification loop as putative acceptor sites for ADP-ribose [150]. Two additional PARP1 mutants were subsequently tested for auto-modification activity. While the double mutant K498/521R still showed residual auto-modification activity, the activity of the double mutant K521/524R was more drastically impaired (Figure 7B). Therefore, K521 and K524 are primary targets for auto-modification.

Besides auto-modification, PARP1 also catalyzes the transfer of ADP-ribose moieties onto PAR acceptor proteins. To test whether lysine residues might also be the target amino acids for trans-ribosylation, poly-L-lysine, poly-L-arginine, and poly-L-glutamate were coupled to cyanogen bromide-activated beads. The beads were incubated with purified PARP1 in the presence of ^{32}P-NAD^+. After removal of PARP1 and free NAD^+, the labeling was determined by measuring scintillation counts. Interestingly, poly-L-glutamate was not a target for trans-ribosylation by PARP1 under the tested conditions (Figure 7C). Instead, poly-L-lysine and, to a lesser extend, poly-L-arginine became modified by PARP1. This finding supports our published conclusions that glutamic acid residues are not the target sites for covalent ADP-ribose attachment by PARP1, but that instead lysine residues are enzymatically modified by ADP-ribose via Schiff base formation [150].

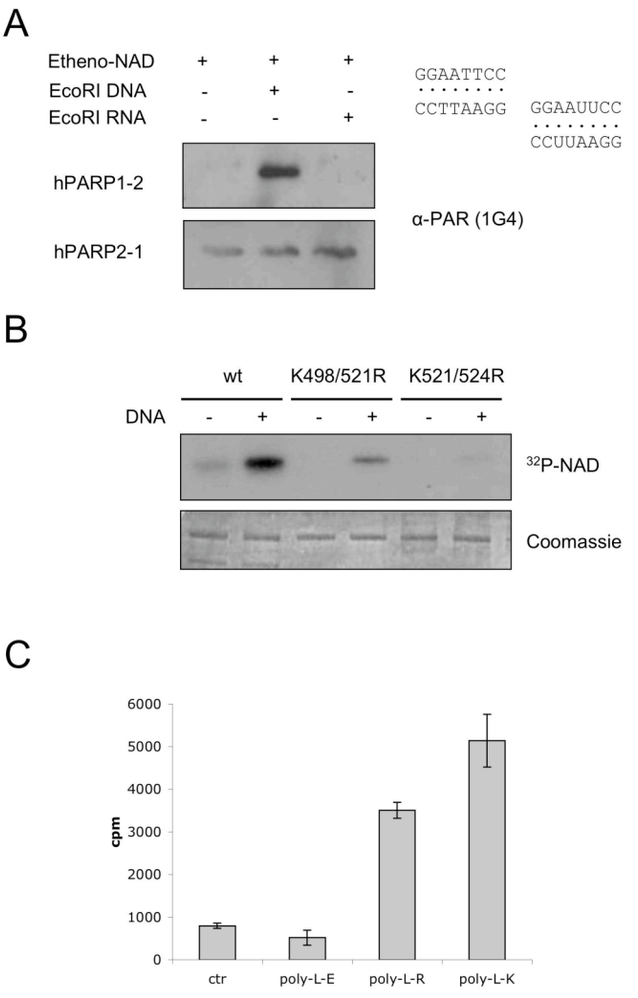


Figure 7: The DBD of PARP1 is activated by double-stranded DNA, but not by RNA, and PARP1 modifies basic amino acids. (A) The enzymatic activity of 10 pmol of the protein chimera hPARP1-2 is activated by 5 pmol of a double stranded DNA oligonucleotide but not by 5 pmol of a double stranded RNA of comparable sequence. Reactions were performed as described previously [150]. (B) hPARP1 with mutations of the amino-acids K498, K521, and K524 in the auto-modification loop has impaired auto-modification activity. 10 pmol of each purified protein was incubated with 100nM ^{32}P-NAD as described previously [150]. (C) Poly-L-arginine and poly-L-lysine but not poly-L-glutamate are modified by hPARP1. Poly-L-amino acids were coupled onto cyanogen-bromide activated agarose beads over night as suggested by the provider (Sigma-Aldrich). Excess poly-L-amino acids were washed away and unoccupied reactive sites were blocked over night. The beads were washed and equilibrated in PARP1 reaction buffer. Reactions were performed for 5 minutes at 30°C in the presence of 100nM ^{32}P-NAD. The beads were washed 3 times in PARP1 reaction buffer containing 500mM NaCl before scintillation counts in two different channels were determined.

2.4.2 PARP1 modifies lysine residues in core histone tails

Histones are known acceptors for ADP-ribose, however single acceptor amino acids have not been identified by either site-directed mutagenesis or mass spectrometry. Having established that lysine residues are the targets for ADP-ribose attachment by PARP1, we tested whether the amino-terminal core histone tails, which are extremely rich in basic amino acids (Figures 8A and B), are substrates for PARP1-mediated ADP-ribosylation. The four core histone tails of H2A, H2B, H3 and H4 were expressed as GST-fusion proteins in bacteria and subsequently purified over glutathione beads. All four tails but not GST alone were labeled by PARP1 *in vitro* (Figure 8C). Modification of the histone tails was not observed when the tails had been added after termination of the reaction with the PARP inhibitor 3-aminobenzamide, strongly indicating that the observed labeling represents covalent modification rather than a non-covalent binding to PAR chains. Furthermore, to demonstrate that the modification of histone tails was catalyzed by PARP1 and not due to non-enzymatic ADP-ribose attachment or due to co-purified NAD^+-consuming enzymes, two PARP inhibitors (PJ34 and DAM-TIQ-A) were used. Both inhibitors blocked both PARP1 auto-modification and modification of the H2B tail (Figure 8D). This finding strongly indicates that histone tail modification by PARP1 occurs via an enzymatic reaction and not by non-enzymatic chemical modification. The tails of H2A, H3 and H4 do not contain glutamic acid residues. The tail of H2B, however, harbors a single glutamate at position 2, which had been proposed to function as acceptor for ADP-ribose [151]. To directly test whether this glutamate is an acceptor site for ADP-ribose, we generated an H2B E2A mutant. This mutant was as efficiently trans-ribosylated by PARP1 as the wild type control (Figure 8E). Again, this finding suggests that also in the H2B tail glutamate 2 is not an acceptor site for ADP-ribosylation synthesized by PARP1.

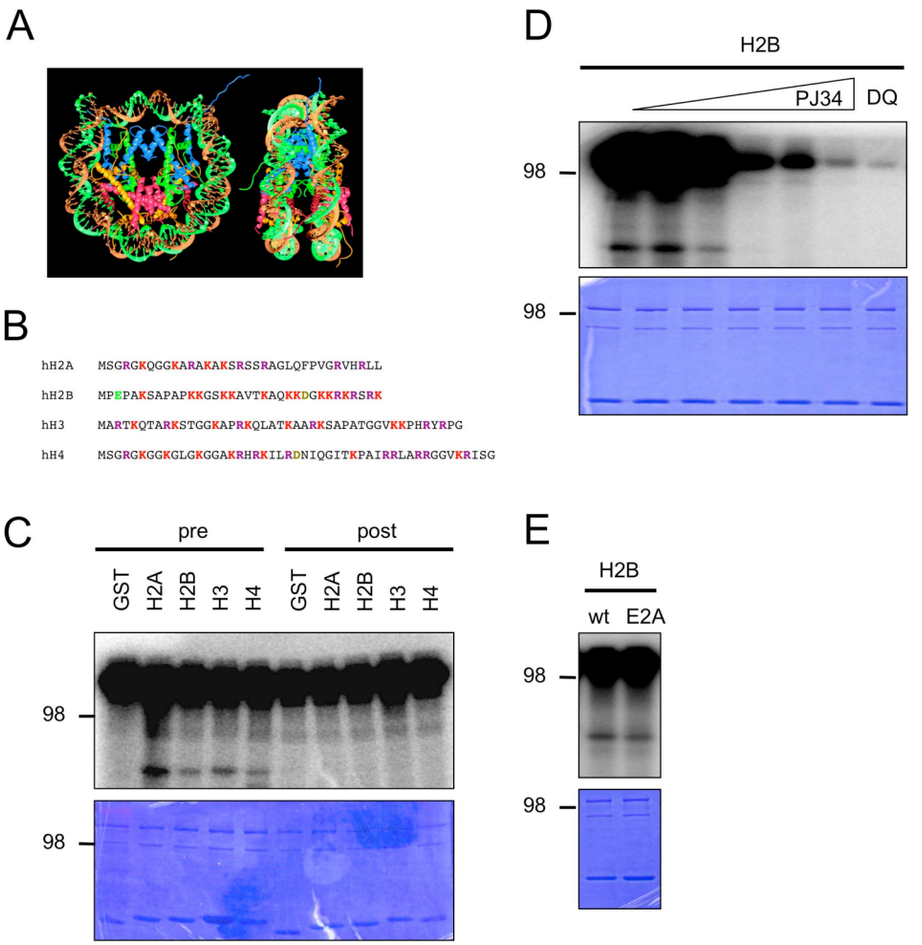


Figure 8: The amino-terminal basic core histone tails are covalently modified by PARP1. (A) Crystal structure of the nucleosome core particle. Blue: H3; green: H4; yellow: H2A; red: H2B; [152]. (B) Amino acid sequence of the amino-terminal human core histone tails. (C) Core histone tails were cloned into pGEX vectors, expressed in bacteria as GST-fusion proteins and purified using glutathione sepharose according to the provided instructions (GE Healthcare). 1.5µg of each purified GST-histone tail were used in PARP1 mediated trans-ADP-ribosylation reactions for 5 minutes at 30°C as described previously [150]. Histone tails were either included during the reaction (pre) or added after the reaction had been stopped with a 100-fold excess of 3-aminobenzamide over ^{32}P-NAD (post) to exclude non-covalent interaction of the histone tails with PAR. (D) Trans-ADP-ribosylation of the H2B tail is inhibited by the PARP inhibitors PJ34 (0.01µM-100µM) and DAM-TIQ-A (10µM). (E) Trans-ADP-ribosylation of H2B is not impaired in an H2B E2A mutant, in which the only glutamic acid residue is substituted by an alanine. Shown are autoradiographies and coomassie stainings.

In order to confine the regions within the core histone tails, which are required for PARP1-mediated ADP-ribosylation, we generated a set of tail deletion mutants (Figure 9A). Successive shortening of the histone tails resulted in loss of ADP-ribosylation (Figure 9B-E).

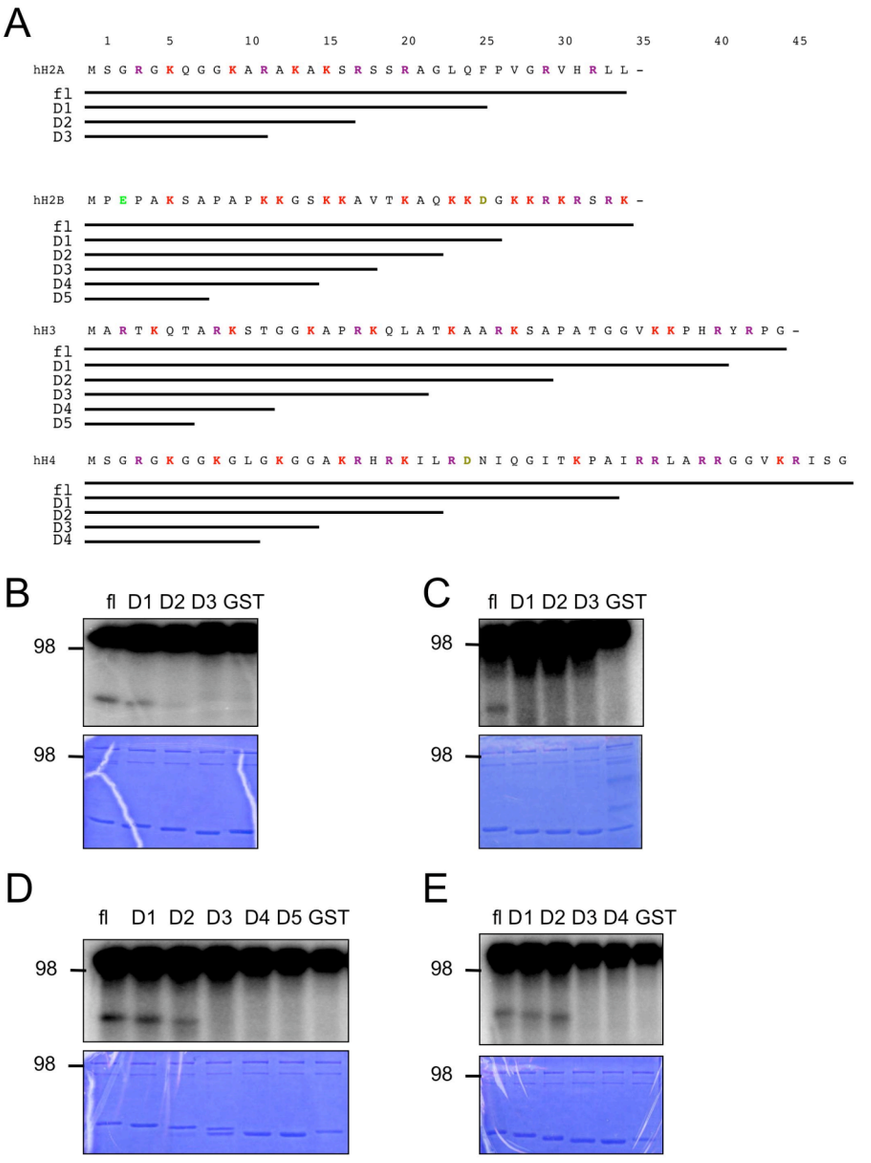


Figure 9: Successive shortening of histone tails leads to loss of trans-ADP-ribosylation by PARP1. (A) Schematic representation of the histone tail deletion mutants, which were generated, expressed and purified. (B) Trans-ADP-ribosylation of H2A deletion mutants by PARP1. (C) Trans-ADP-ribosylation of H2B deletion mutants by PARP1. (D) Trans-ADP-ribosylation of H3 deletion mutants by PARP1. (E) Trans-ADP-ribosylation of H4 deletion mutants by PARP1. Shown are autoradiographies and coomassie stainings.

The shortest histone tail deletion mutant, which was still modified by PARP1 was used to generate single and combinational lysine and arginine mutations. Following this approach, for H2A the lysines at position 13 and 15, and an arginine at position 20 were identified as putative PAR acceptor sites and/or PARP1 interaction sites (Figure 10A). For H2B, seven lysines and arginines between position 27 and 34 had to be mutated in order to lose modification completely (Figure 10B).

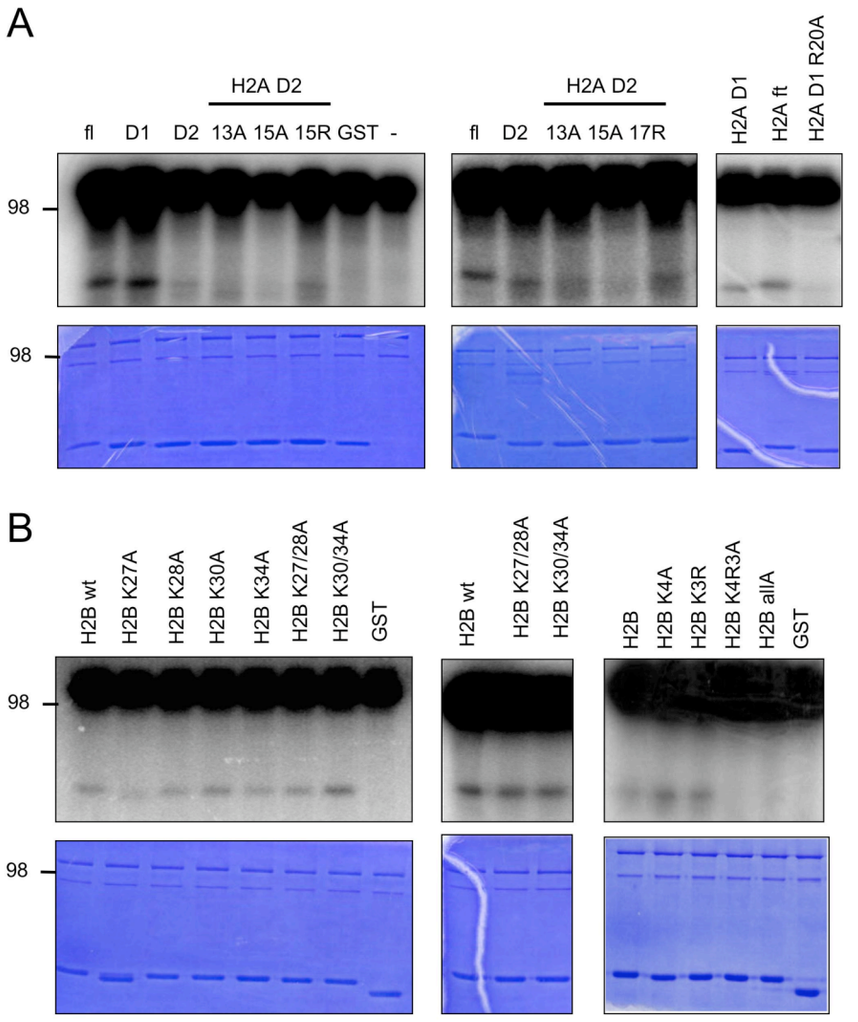


Figure 10: Point mutations of lysine and/or arginine residues in the tails of H2A and H2B impair trans-ADP-ribosylation by PARP1. (A) The indicated wild type or mutant H2A tails were used in PARP1 mediated trans-ADP-ribosylation assays. (B) The indicated wild type or mutant H2B tails were used in PARP1 mediated trans-ADP-ribosylation assays. Shown are autoradiographies and coomassie stainings.

Mutagenesis of the H3 tail revealed putative PAR acceptor sites and/or PARP1 interaction sites at lysines 23 and 27, and at arginine 26 (Figure 11A). For histone H4, mutation of lysines 16 and 20, and of arginines 17 and 19 resulted in severely reduced modification by PARP1 (Figure 11B).

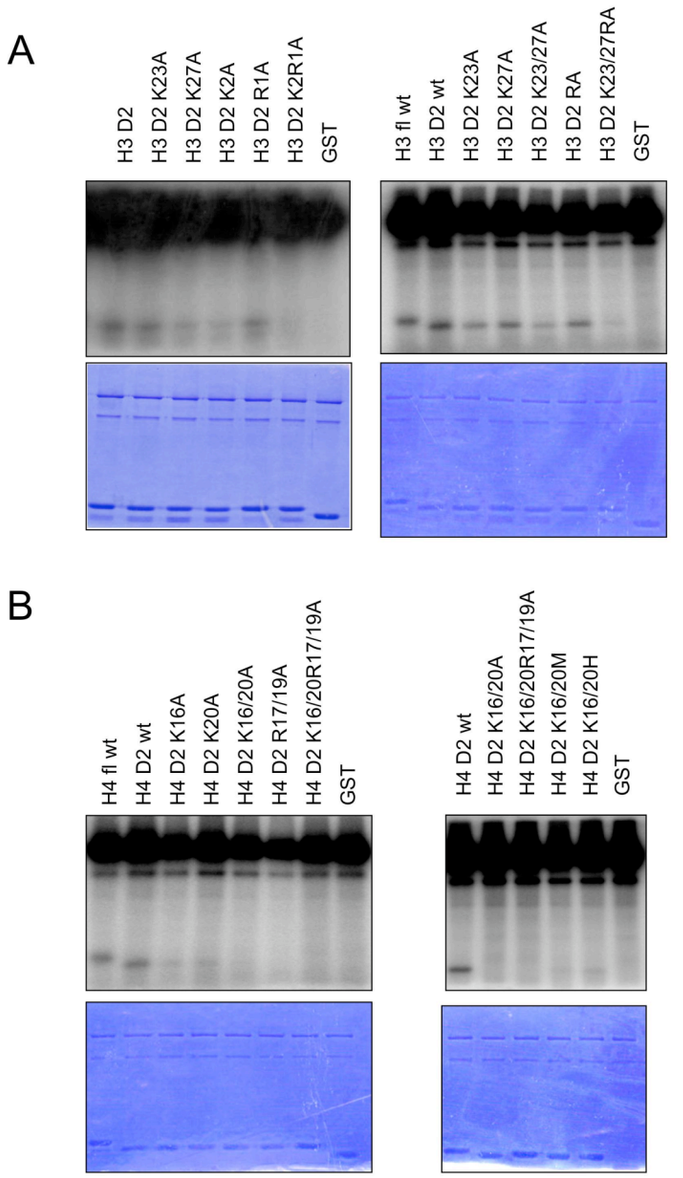


Figure 11: Point mutations of lysine and/or arginine residues in the tails of H3 and H4 impair trans-ADP-ribosylation by PARP1. (A) The indicated wild type or mutant H3 tails were used in PARP1 mediated trans-ADP-ribosylation assays. (B) The indicated wild type or mutant H4 tails were used in PARP1 mediated trans-ADP-ribosylation assays. Shown are autoradiographies and coomassie stainings.

Interestingly, the identified regions in the four core histone tails, which are required for PARP1 mediated ADP-ribosylation, overlap with the regions where the tails exit the nucleosomal core (Figures 12A and B).

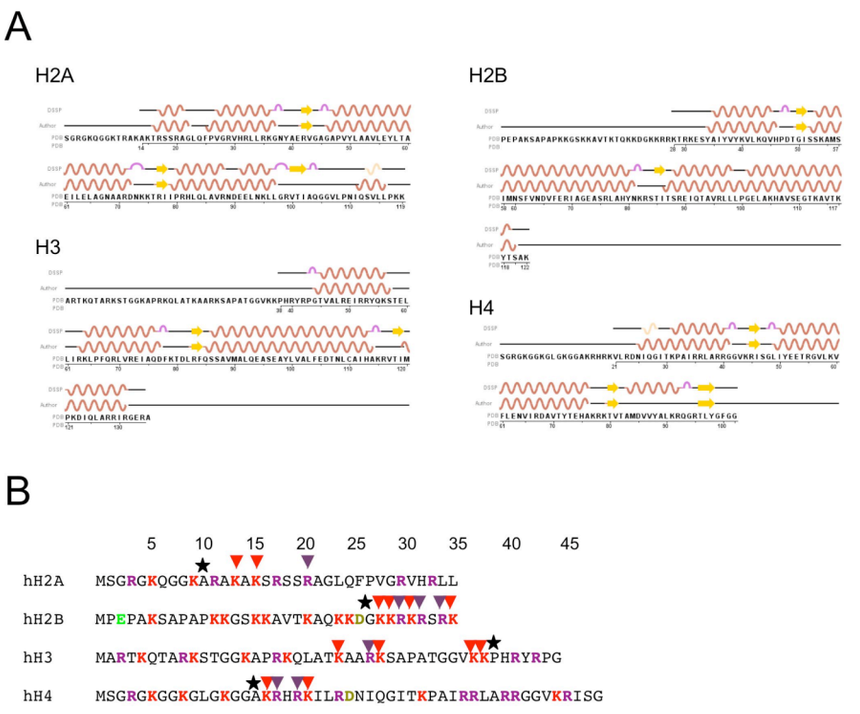


Figure 12: Summary of identified putative ADP-ribosylation sites in core histone tails. (A) Schematic representation of the secondary structure of the four core histones (PDB: 2NZD). (B) Identified putative PARP1 target sites in the amino-terminal tails of the four core histones. Lysines are marked in red, arginines in purple. Asterisks indicate the approximate point where the tails exit the DNA superhelical gyres to the exterior of the nucleosome [153].

Remarkably, a structural modeling for PARP1`s catalytic domain and the tail of H4 revealed nice fitting of the histone tail into the major pocket of the PARRP1 catalytic domain, bringing the putative ADP-ribose acceptor lysines close to the catalytic core (Figure 13A). Furthermore, in line with the finding that PARP2 is not able to modify histone tails (Simon Messner, unpublished results), the structural modeling revealed that an additional loop present in the PARP2 catalytic domain would displace the H4 tail from the catalytic pocket (Figure 13B). Together, these data demonstrate that (1) the amino-terminal histone tails of all four core histones are targets for covalent modification by PARP1 at specific lysine residues, (2) that histone modification is specific for PARP1 because an additional loop within the PARP2 catalytic domain

impedes histone tail binding, and (3) glutamic acid residues are not required for trans-ribosylation of histone tails and are not the target sites for ADP-ribose attachment.

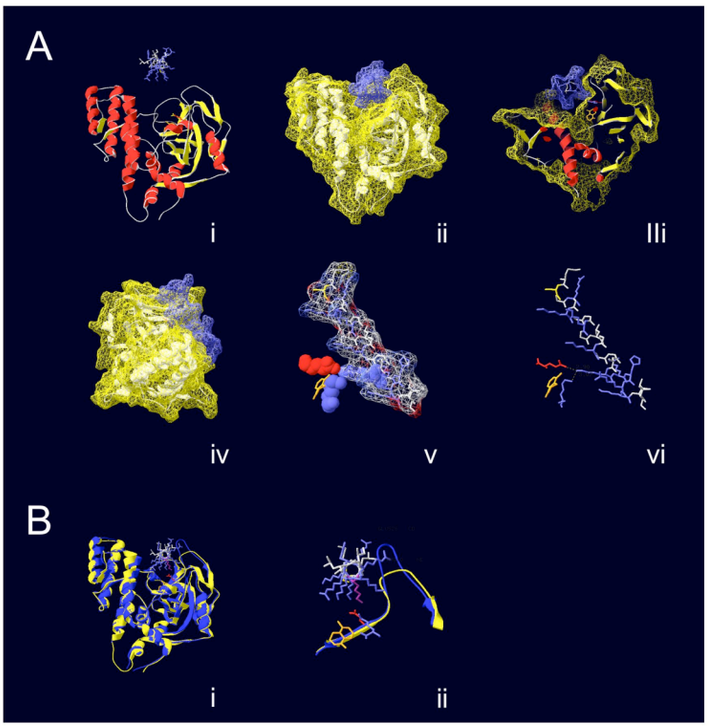


Figure 10: The histone H4 tail fits into the catalytic cleft of PARP1. (A) The H4 tail (aa 1-22) was modeled into the catalytic cleft of PARP1. PDB files were obtained from RCSB PDB (www.rcsb.org/pdb/home/home.do), the modeling was performed using the Swiss-PDBViewer. Molecular surfaces were calculated and used as constraints for model making. The H4 tail was arranged in a way that H4 K16 comes into close proximity to PARP1 E988, K903 and the substrate. (B) The catalytic domain of PARP2 was aligned with the catalytic domain of PARP1 using the Magic Fit function of the Swiss-PDBViewer. An additional loop of PARP2 is not compatible with H4 binding.

2.4.3 PARP1 modulates pro-inflammatory gene expression

PARP1 is a known co-regulator for NF-κB target gene expression [3]. Which subset of NF-κB target genes requires PARP1 for efficient transcription and how exactly PARP1 influences pro-inflammatory gene expression is only poorly understood. We thus set out to identify and characterize the subset of PARP1-dependent NF-κB target genes using customized NF-κB target gene microarrays and quantitative RT-PCR (qPCR). Raw264.7 macrophages were treated with LPS for 4 hours before total RNA was isolated and used for microarray analysis. LPS treatment resulted in the up-regulation of 211 NF-κB target or NF-κB related genes (of a total number of 524 genes covered by the array) at least 1.6-fold (Figure 14A). Interestingly, pre-treatment of cells with the potent PARP inhibitor DAM-TIQ-A, which blocks genotoxic stress induced PAR formation (data not shown), did not significantly alter the gene expression profile, suggesting that PAR formation is not a prerequisite for NF-κB target gene expression (Figure 14A). PARP1 knockdown by shRNA (Figure 14B), on the other hand, dramatically changed the gene expression profile after LPS treatment (Figure 14C). Overall, gene induction after LPS treatment was reduced in shPARP1 cells and gene repression after LPS treatment was increased. More specifically, the microarray data revealed that the induction of several NF-κB target genes, including Mpa2l, Ccl7, MIP-2, and IκBε, was impaired in Raw264.7 cells lacking PARP1 (Figure 14D). Other NF-κB targets such as IL-6 were induced independently of PARP1. These data support a transcriptional co-activator function of PARP1 for a subset of NF-κB target genes. In order to confirm the microarray results by qPCR, we followed the gene expression of Mpa2l at different time-points after LPS treatment. A time-dependent gene induction was observed, which was markedly reduced in shPARP1 cells (Figure 14E). Furthermore, the microarray data was also confirmed for other genes by qPCR (Figure 14F). Together, these findings indicate that PARP1 is required for the efficient expression of a subset of NF-κB target genes after LPS treatment of Raw264.7 macrophages.

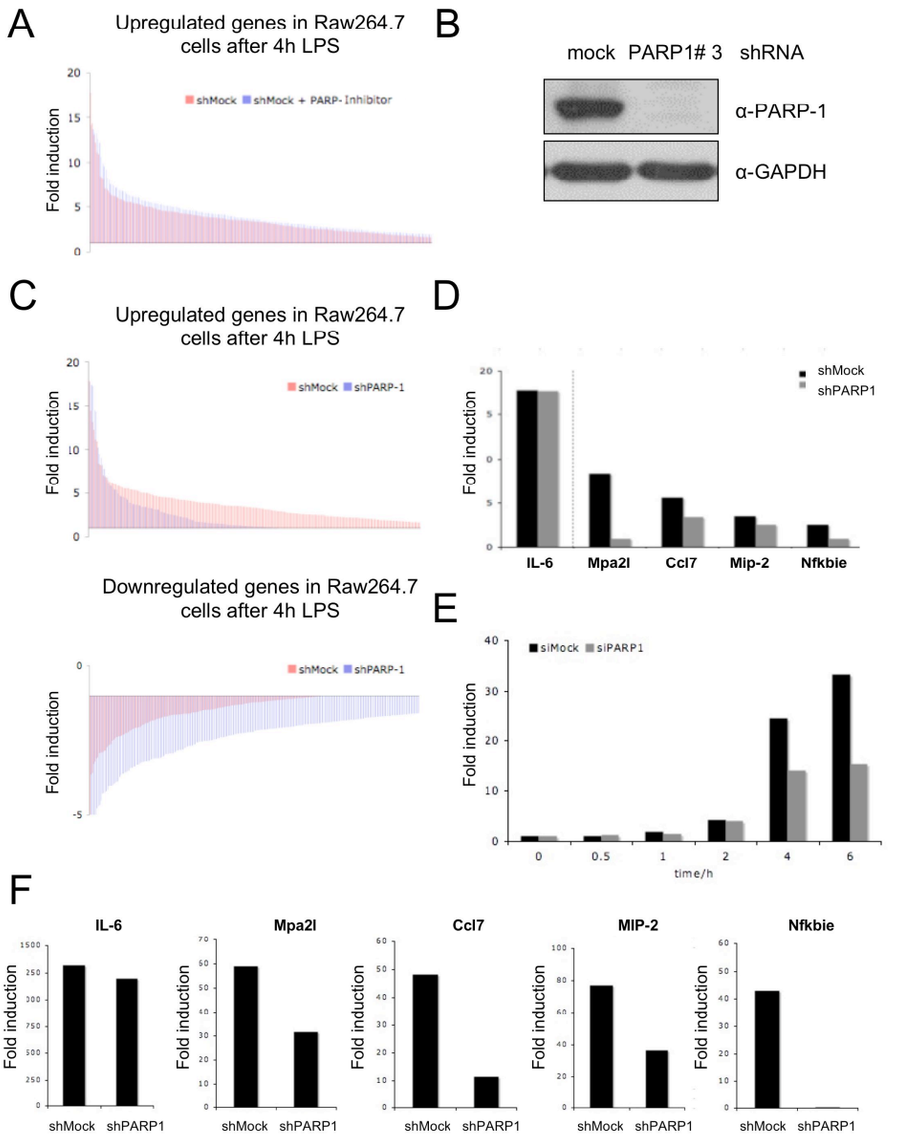


Figure 14: PARP1 influences LPS-induced NF-κB target gene expression in Raw264.7 macrophages. (A) Treatment of PARP1 proficient cells with 10μM PARP inhibitor DAM-TIQ-A 1 hour prior to treatment with 10μg/ml LPS for 4 hours has no gross effect on upregulated gene expression as revealed by customized microarray analysis for NF-κB target genes. (B) PARP1 was stably depleted from Raw264.7 macrophages by shRNA and protein levels were analyzed by Western blot. (C) Depletion of PARP1 by shRNA reduces the number of upregulated genes after LPS stimulation (upper panel) and increases the number of downregulated genes after LPS stimulation (lower panel) as revealed by customized microarray analysis. Each bar represents a single gene probe on the customized microarray. Fold changes after LPS stimulation are shown and genes are ranked by fold change. (D) Microarray results for single genes comparing PARP1 deficient and control cells. (E) Time-course of Mpa2l gene expression after LPS treatment in PARP1 deficient and control cells as revealed by quantitative RT-PCR. (F) Validation of microarray results for single genes 4 hours after LPS treatment by quantitative RT-PCR.

Next, we analyzed PARP1-dependent gene expression in peritoneal macrophages isolated from wild type or PARP1 knockout mice. Cells isolated from wild type animals generated PAR in response to oxidative stress, and PAR formation was efficiently blocked by the PARP inhibitor DAM-TIQ-A (Figure 15A). Cells isolated from PARP1 knockout mice, on the other hand, did not synthesize PAR in response to oxidative stress (Figure 15B).

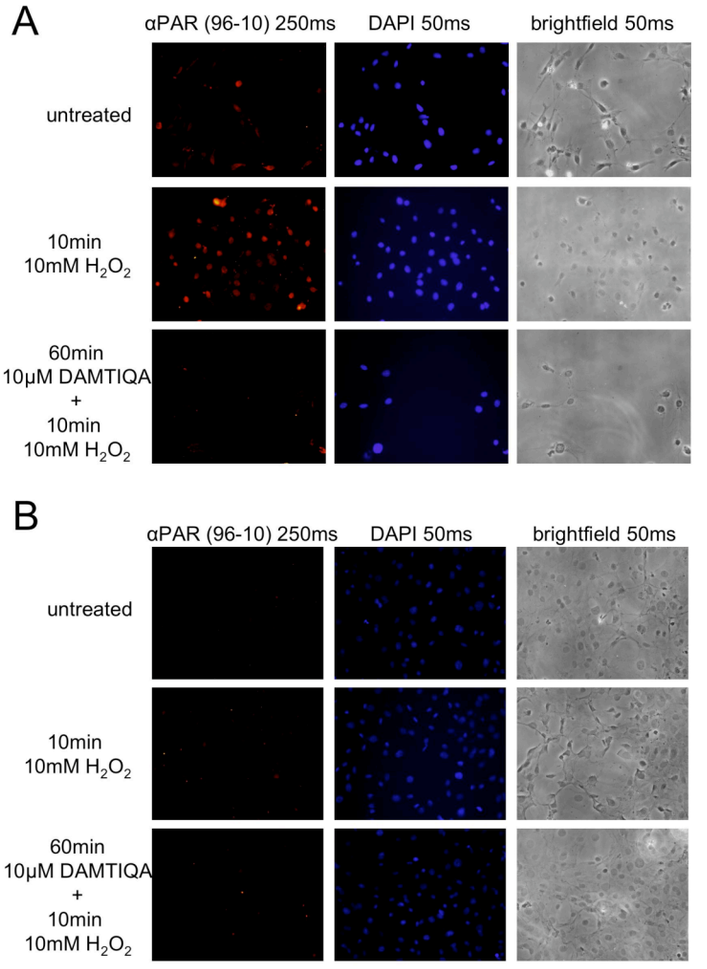


Figure 15: PARP inhibition reduces PAR formation in peritoneal macrophages after H_2O_2 treatment. Peritoneal macrophages were treated as indicated and PAR formation was analyzed by immunofluorescence. Macrophages had been isolated from wild type (A) or PARP1 knockout mice in the 129S background (B).

Following LPS treatment, both cell lines expressed IL-10, IκBα, and COX-2 and the induction of these genes was independent of PAR formation (Figure 16A). We confirmed that PARP1 was expressed in wild type but not PARP1 knockout cells by

Western blot (Figure 16B), before we used customized microarrays (Figure 16C) to analyze NF-κB target gene expression after LPS treatment. 4 hours after LPS treatment, several genes were induced at least 2-fold (Figure 16D). Whereas some genes were expressed independent of PARP1, others showed impaired induction in PARP1 knockout cells. As in Raw264.7 cells, IL-6 was PARP1-independent, whereas Mpa2l, MIP-2 and IκBε were PARP1-dependent. Other PARP1-dependent genes include Vcam1, Icam1, COX-2 and Aif1.

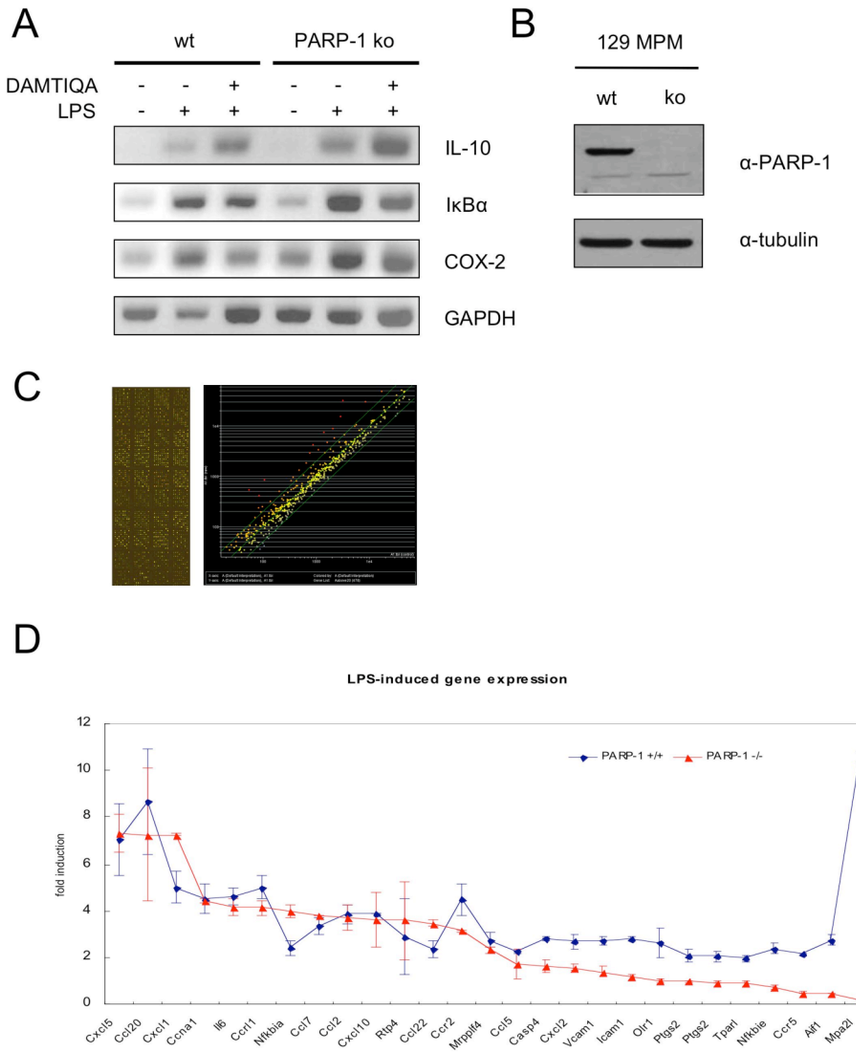


Figure 16: PARP1 influences LPS-induced NF-κB target gene expression in peritoneal macrophages. (A) LPS induced gene expression 4 hours after treatment as revealed by conventional RT-PCR. (B) PARP1 protein levels in cells isolated from wild type or PARP1 knockout mice as revealed by Western blot. (C) Representative example of the customized microarray analysis to study NF-κB target gene expression. (D) Microarray results comparing wild type and PARP1 knockout cells. Genes with a more than 2-fold induction in wild type cells after LPS treatment are shown.

From the discussed analyses, the three genes Aif1, Mpa2l and IκBε were robustly PARP1-dependent in two different cell culture systems. We confirmed PARP1-dependency of these genes also *in vivo*, as all three genes were less induced in gastric epithelium isolated from PARP1 ko mice four weeks after infection with *Helicobacter felis* when compared to *H. felis* infected wild type mice (data not shown). Thus, from gene expression analyses of three different biological conditions (LPS treated Raw264.7 cells, LPS treated peritoneal macrophages, *H. felis* infected mice) the three genes Aif1, Mpa2l and the negative feedback regulator of NF-κB, IκBε, were most robustly dependent on PARP1 for their expression.

In an attempt to define what makes a gene PARP1-dependent, we used a bioinformatics approach to analyze PARP1-dependent versus PARP1-independent genes. When comparing PARP1-dependent with PARP1-independent genes as revealed by microarray analyses of LPS stimulated peritoneal macrophages, there was no difference in occurence of upstream *cis* elements or TATA box sequences (200 / 1000 bp upstream and 1000 bp downstream of transcription start) (Figure 17A), no difference in chromosomal localization (chromosome number, distance to telomeres/centromere, +/- strand) and no difference in average gene length (Figure 17B). Furthermore, there was no difference in the occurence of alternative splicing, gene overlaps or bidirectional promotors (data not shown) and no difference in the majority of pathways the genes are involved in (immunological disease, inflammatory disease, immune response, cancer, cellular movement, cell signaling). A significant difference was observed, however, in the involvement in cell death (8/9 vs. 4/11) and development and function of the reproductive system (5/9 vs. 1/11) (Figure 17C).

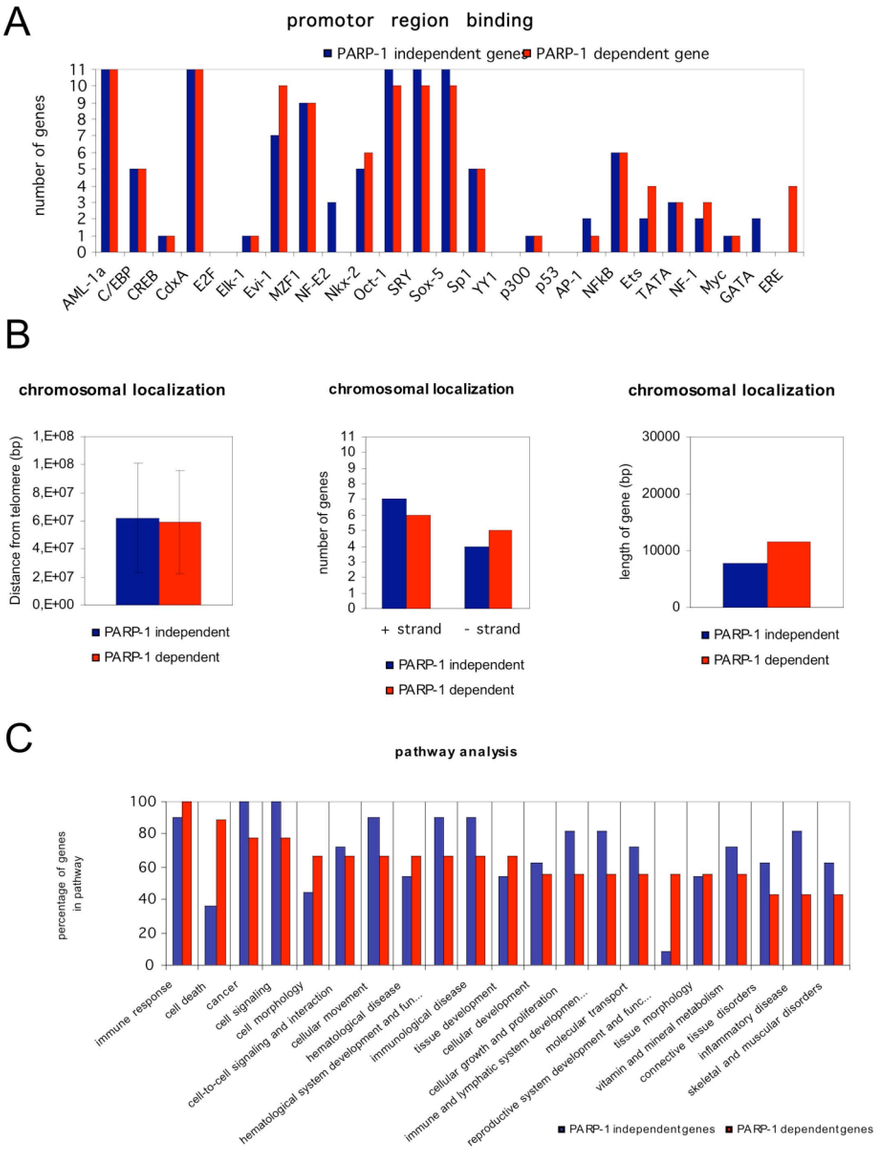


Figure 17: Global analysis of PARP1 dependent genes after LPS treatment of peritoneal macrophages. Based on the microarray analysis, PARP1 dependent versus PARP1 independent genes were analyzed *in silico*. PARP1 dependent genes did not show any obvious change in binding sites for DNA binding proteins (P-Match: http://www.gene-regulation.com/cgi-bin/pub/programs/pmatch/bin/p-match.cgi.) (A), distance from telomeres, orientation, or gene length (B), nor in the pathways they are involved in (Ingenuity Pathways Analysis, Ingenuity® Systems, www.ingenuity.com) (C).

A slight difference was also observed at the positions 3, 8 and 10 of the NF-κB consensus binding sequence (κB-site) (Figure 18A) and in the predicted nucleosome formation potential specifically around the transcription start site (TSS) but not upstream or downstream of the TSS (Figure 18B).

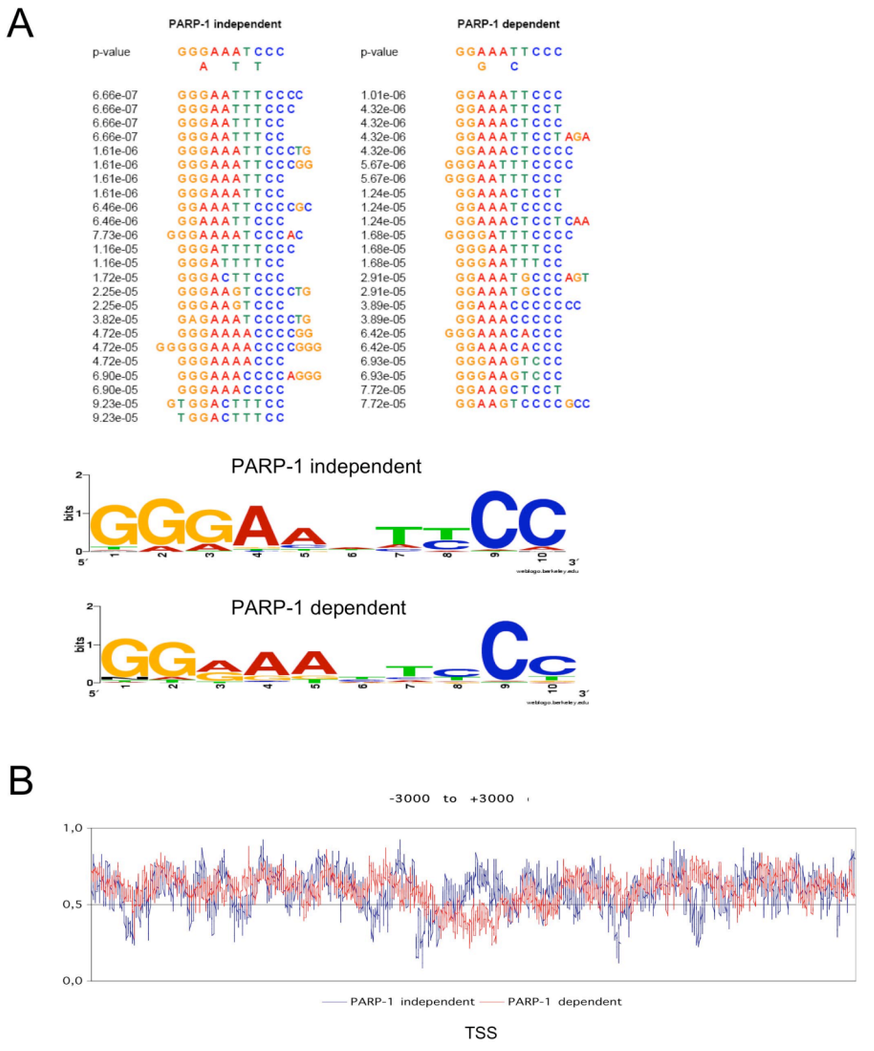


Figure 18: Global analysis of PARP1-dependent genes after LPS treatment of peritoneal macrophages. Based on the microarray analysis, PARP1-dependent versus PARP1-independent genes were analyzed *in silico*. PARP1-dependent genes showed a slight difference in the κB-site consensus sequence at positions 3, 8 and 10 (http://meme.sdsc.edu/meme/intro.html) (A) and in the nucleosome formation potential around the transcription start site (TSS) [154] (B).

PARP1 had recently been suggested to bind to S/MAR elements of certain genes [155, 156], but no difference was observed in S/MAR element-typical stress-induced duplex destabilization (SIDD) profiles between PARP1-independent and PARP1-dependent genes (Figures 19A and B).

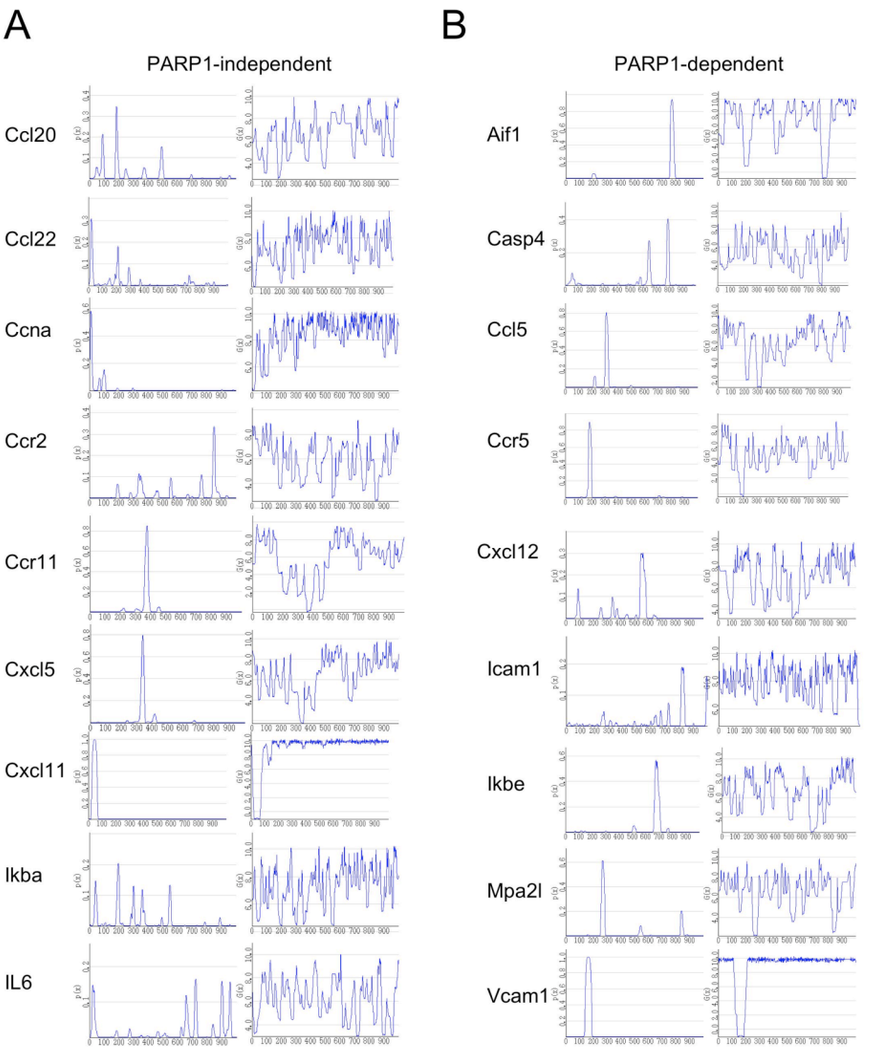


Figure 19: (A, B) Global analysis of PARP1 dependent genes after LPS treatment of peritoneal macrophages. Based on the microarray analysis, PARP1 dependent versus PARP1 independent genes were analyzed *in silico*. PARP1 dependent genes showed no difference in stress-induced duplex destabilization (SIDD) profiles [155, 156]. Probabilities p(x) and G(x) are depicted for genomic sequences from -1000 bp upstream of the TSS to the TSS. http://www.genomecenter.ucdavis.edu/benham/sidd/

Very recently, in a large scale screen for sequence-specific protein-DNA interactions, the PARP1-interacting protein XRCC1 was found to bind DNA in a sequence-specific manner [157]. Interestingly, we identified an XRCC1 consensus binding site just 450 bp upstream of the TSS of IκBε, one of the genes, whose expression most robustly depended on PARP1 (Figure 20). XRCC1 consensus binding sites or variations thereof were also found in the promotor regions of the PARP1-dependent genes Icam1, Ccr5, Casp4, Mpa2l, Ccl5, Vcam1 and Olr1, but not in the promotor regions of PARP1-independent genes (data not shown). Together, these analyses revealed variations in κB-sites, differences in nucleosome formation potential around the TSS and differences in XRCC1 binding sites in the promotor regions as possible characteristics to differentiate between PARP1-dependent and PARP1-independent genes. Especially the finding that PARP1-dependent genes contain XRCC1 binding motifs is intriguing and it will be interesting to investigate the putative role of the DNA damage repair protein XRCC1 for PARP1-dependent gene expression.

>Nfkbie_ref|NT_039649.7|Mm17_39689_37:31885451-31886451 Mus musculus chromosome 17 genomic contig, strain C57BL/6J
TCTATCATCAGGGGAGTTGACCCAGAACTGTCTATGAAGGTTTCCCAGGGAAAAGCTGCTCCCCTGAAGA
TAATAGATGCACTCTCTCTTACCAATCCCCCTGAGCACTGATTGAAAATAAGTGAAAAGGCAAGAGAAGT
TGAGAAAAAAATAGGCAGAGGATAGCAAGCAGAGCCACCATCACCACCCCTTCCCCATCTGTGCTGGCTA
GTCAGGCAACTTGATGGGAGCCAGCGTCATCTGAAAAGAGGGAACCTCGGTTAAAATGCCTCCATAAGAT
CCAGCTGTAAGCCTCTTTCTTAATTAGTGACTGATGGGGAAGGCCCACCCCATCCCATTGTGCATGATGC
CATCCCTGGGCTGGTATTCCTGGGTTCTATAAGAAAGCAGGCTGAGCAAGCCATGGGGAGCAAGCCAGTG
TGCAAGACGCTCCCCATGGCCTCTGCATCAGCTTCTCCCTCCAGGATCATGTCCTGTTTCAGTTTTGTCC
TGACTTCCTTTGATAATGGATTATGATCTTGAAGTATAAGCCGAATAAAACCTTCTCTCCCCAATTTGCT
TTTTGGTCAGGGTATTTCATTGCAGCAATAGAAACGCTAAGACACCCCACCACCACCACCAACCTACACA
AACTCCCCACAAAGTAGCCGGTTTATGCAGAAAAGTTTTTTAAAAATACACTTTTTAAACAATTGAATGA
ATAAATGAATGAAGACACCACGGAACTAAAAAGAAGGAACAAACAAATGCACGGAATTAGTGAATGAAAT
AAAAGGGAAGGGGAAGAATGAGTGAATGAATGAATGGGCGAAACAATCCACGCAGGAGTGAGTCAAGACT
GGTAACCAGAGACTCCCTGCCTACGCAAGCAGCAAACAGGGGGATTCCGGAGGGGCGGGGCTCTACCCGG
AAGCTCCTTTGAGGGCGGGGCTGTGGGCGTGGCAGTGGCTGCGGGCCACTTCTTGGGTCCGGGGGCGGGG
CGACAGAGAAGACCTAACTA

XRCC1 consensus sequence (PWM):

Figure 20: An XRCC1 consensus binding motif is present 450 bp upstream of the TSS of IκBε. The genomic sequence from -1000 bp upstream of the TSS to the TSS of IκBε is shown. The XRCC1 consensus sequence as previously described [157] is boxed and shown below as positional weight matrix (PWM).

2.4.4 PARP1 is activated by *Helicobacter pylori*

In collaboration with Isabella Toller and Anne Müller (both IMCR, UZH) a possible role for PARP1 in *Helicobacter pylori* induced gastric inflammation was investigated (manuscript in preparation). We used the transformed gastric epithelial AGS cell line to study the potential interplay between PARP1 and *Helicobacter pylori.* PARP1 was expressed in this cell line as revealed by immunofluorescence (Figure 21A). In order to study PARP1 function, PARP1 was depleted from AGS cells by shRNA (Figure 21B). PARP1 knockdown had no effect on the morphological changes typically observed after infection of AGS cells with *H. pylori* (Figure 21C).

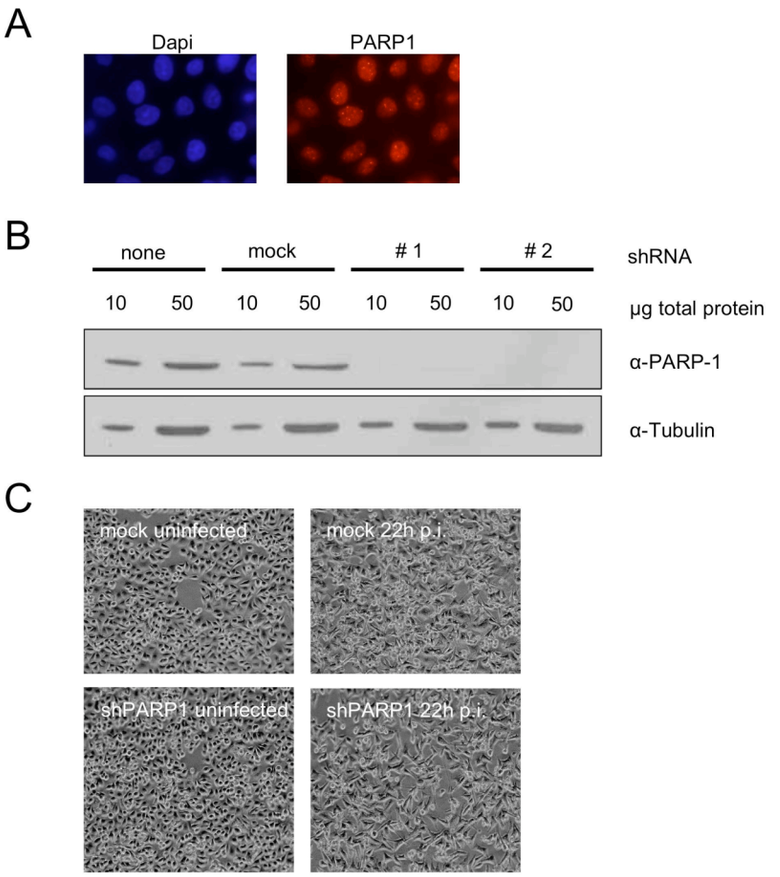


Figure 21: *Helicobacter pylori* induces morphological changes in AGS cells independent of PARP1. (A) PARP1 is expressed in AGS cells as revealed by immunofluorescence. (B) PARP1 was stably depleted from AGS cells by two different shRNAs and protein levels were determined by Western blot. (C) AGS cells were infected with *Helicobacter pylori* and morphological changes were recorded 22 hours p.i. by light microscopy.

Unexpectedly, a protein at the size of about 55kDa was detected with a homemade anti-PARP1 antibody after *H. pylori* infection (Figure 22A). This protein was induced independent of the presence of PARP1. The protein was not observed in *H. pylori* lysates (data not shown) and therefore most likely was expressed by AGS cells and not by the bacteria. The protein was not analyzed any further nor identified by mass spectrometry. *H. pylori* infection did not alter total cell NF-κB protein levels (Figure 22B).

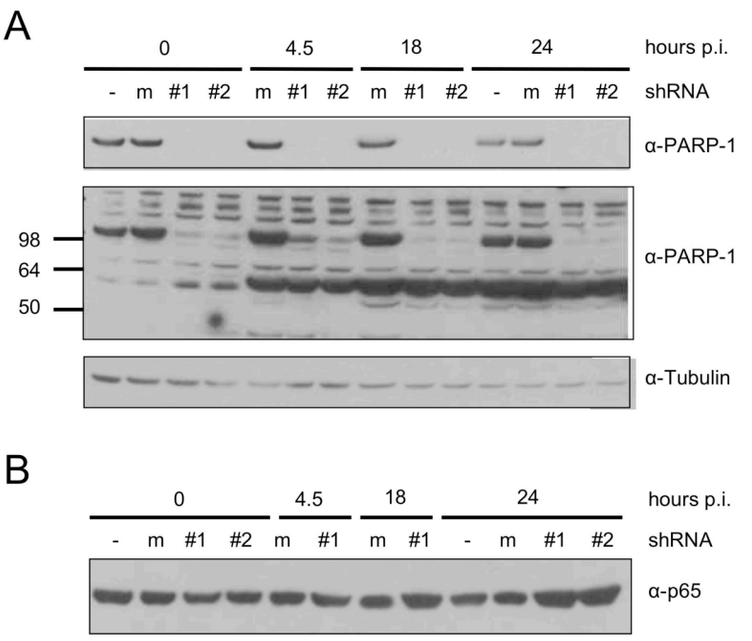


Figure 22: *Helicobacter pylori* induces an unidentified protein of about 55kDa. (A) Western blot analysis of AGS whole cell extracts. (B) p65 protein levels are unchanged after *Helicobacter pylori* infection as revealed by Western blot.

Interestingly, infection of AGS cells with *H. pylori* resulted in the time-dependent synthesis of PAR (Figure 23A). PAR synthesis was blocked when cells were co-treated with the PARP inhibitor PJ34 (Figure 23B). PAR formation after *H. pylori* infection was also observed in immortalized primary gastric epithelial cells (IMPGEs) and also in these cells PAR synthesis was blocked by PJ34 (Figure 23C).

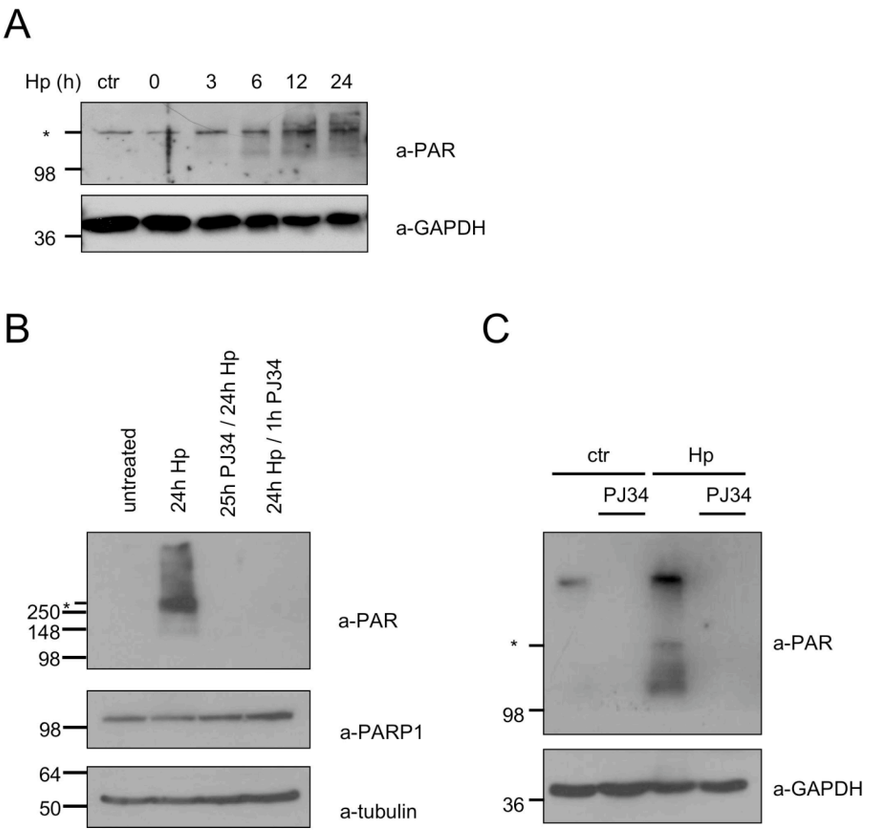


Figure 23: *Helicobacter pylori* induces PAR formation in gastric epithelial cells. (A) *Helicobacter pylori* induces time-dependent PAR formation in AGS cells as revealed by Western blot. (B) *Helicobacter pylori* induced PAR formation is abolished when AGS cells are treated with PARP inhibitor PJ34 (10μM) during infection. (C) *Helicobacter pylori* induces PAR formation 24 hours p.i. in IMPGE cells and PAR formation is abolished by PJ34 treatment.

In order to test whether PARP1 was responsible for *H. pylori*-induced PAR formation, we depleted PARP1 in AGS cells by siRNA or shRNA (Figure 24A). Stable or transient knockdown of PARP1 greatly reduced PAR formation after *H. pylori* treatment, suggesting that indeed PARP1 is the main PARP family member responsible for *H. pylori*-induced PAR synthesis (Figures 24B and C). Remarkably, *H. pylori* mutants lacking the CagA effector protein did not induce PAR formation to the same extend as wild type bacteria (Figures 24C and D). *H. pylori*-induced PAR

formation is unlikely to be associated with apoptosis, as we were unable to detect cleaved PARP1 or cleaved caspase 3, two markers for apoptosis, in *H. pylori* infected cell extracts (Figure 24E). Together, these findings indicate that *H. pylori* infection of gastric epithelial cells results in PARP1 activation and PAR formation as a cellular response, which is not associated with cell death. Moreover, *H. pylori* CagA seems to be involved in PARP1 activation.

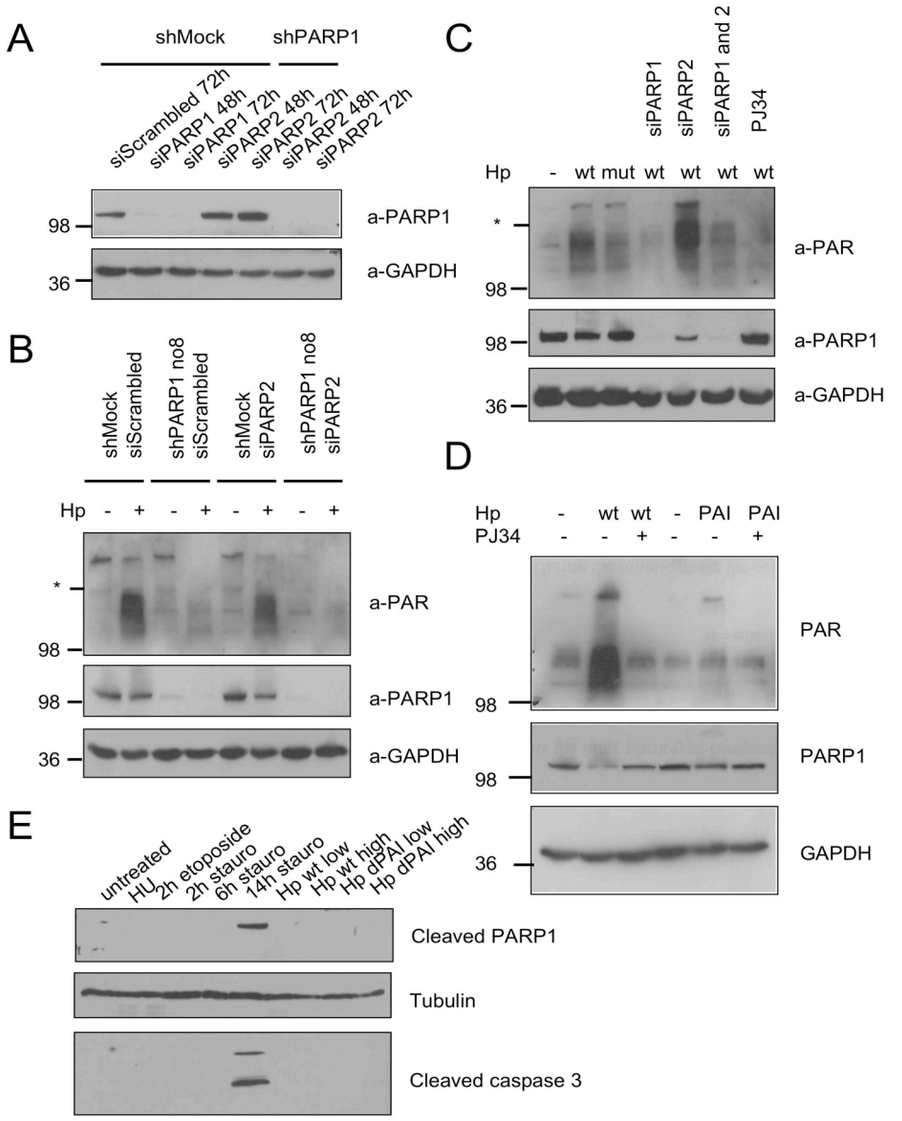


Figure 24: PARP1 is mainly responsible for *Helicobacter pylori* induced PAR formation in AGS cells. (A) PARP1 or PARP2 were transiently depleted from AGS cells by siRNAs. PARP1 protein levels were analyzed by Western blot. (B) PAR formation 24 hours p.i. was analyzed by Western blot in cells stably (shRNA) or transiently (siRNA) depleted for PARP1 or PARP2. (C) PAR formation 24 hours p.i. with wild type (wt) or CagA mutant (mut) *Helicobacter pylori* was analyzed. (D) PAR formation 24 hours p.i. with wild type (wt) or CagA mutant (PAI) *Helicobacter pylori* in the absence or preence of PJ34 was analyzed. (E) *Helicobacter pylori* does not induce significant levels of apoptosis in AGS cells 24 hours p.i. AGS cells were treated as indicated and two markers for apoptosis, cleaved PARP1 and cleaved caspase 3, were analyzed by Western blot.

Since PARP1 is a co-activator for NF-κB target gene expression and *H. pylori* triggers a pro-inflammatory response in the gastric epithelium, we analyzed NF-κB target gene expression in *H. pylori* infected AGS cells. Known NF-κB targets such as IL-8, IL-1b and COX-2 were readily induced by *H. pylori* in PARP1-proficient cells (Figure 25). In cells depleted for PARP1, however, a reduced gene expression was observed. IL-8, IL-1b and COX-2 all showed a 50% reduced expression level in shPARP1 cells, while activation-induced deaminase (AID) was induced normally (Figure 25). Thus, PARP1 is involved in the efficient expression of NF-κB target genes after infection with *H. pylori.*

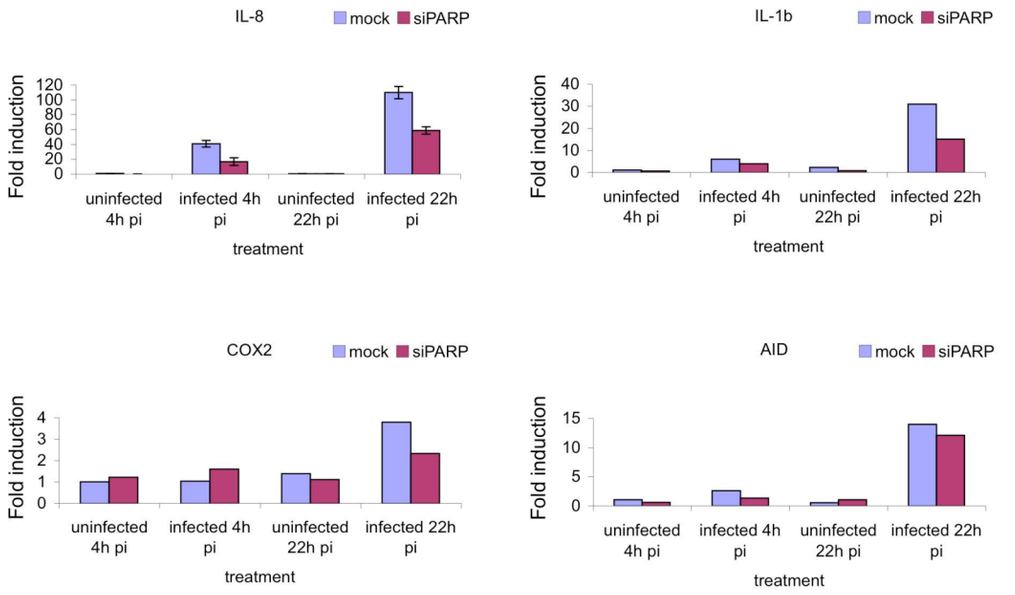


Figure 25: A subset of *Helicobacter pylori* induced genes is PARP1 dependent. Gene expression in control cells and cells depleted for PARP1 was analyzed by quantitative RT-PCR.

2.4.5 PARP1 is required for viral replication

In collaboration with Daniel Mayer and Martin Schwemmle (Virology, University of Freiburg, Germany) the possible role of PARP1 for intracellular replication of RNA viruses was investigated. Mouse lung fibroblasts (MLFs) derived from wild type or PARP1 knockout animals (Figure 26A) were infected with a low MOI of semliki forest virus (SFV) or feline panleukopenia virus (FPV). At different time-points after infection, virus-containing cell supernatants were taken and virus titers were analyzed

by standard plaque assays. Interestingly, SFV titers were 10.000 fold lower in PARP1 knockout MLFs as compared to wild type MLFs (Figure 26B). Importantly, the replication defect observed in PARP1 knockout MLFs could be completely rescued by re-introduction of PARP1, strongly indicating that SFV requires host cell PARP1 for replication. FPV replication, on the other hand, did not require PARP1 (Figure 26C). Together, these data suggest an important so far uncharacterized function for PARP1 in RNA virus replication, which is not observed when DNA viruses are tested (Daniel Mayer, unpublished data).

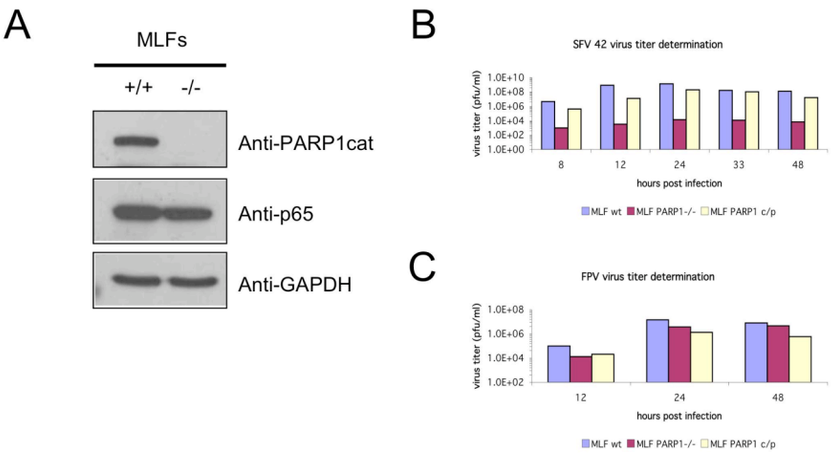


Figure 26: PARP1 is required for SFV replication. (A) Mouse lung fibroblasts (MLFs) from wild type or PARP1 knockout mice were tested for the expression of PARP1, p65 and GAPDH by Western blot. (B) MLFs from wild type or PARP1 knockout mice or MLFs from PARP1 knockout mice, which had been complemented with human PARP1, were infected with SFV using an MOI of 0.1. Virus supernatants were collected at the indicated time-points. Virus titers were determined on confluent Vero cells using serial dilutions of virus supernatants (10^{-2} to 10^{-9}) and standard plaque assays. (C) As in (B) with FPV.

2.4.6 PARP1 expression in mouse spleen

When PARP1 expression in mouse cecum was analyzed by immunofluorescence, we noted PARP1 expression mainly in the proliferative zone of the crypts (Altmeyer et al., submitted manuscript). In order to test whether PARP1 was also differentially expressed in other tissues, we analyzed PARP1 expression in mouse spleens. Interestingly, also in spleens only a subpopulation of cells expressed PARP1 as revealed by immunofluorescence (Figure 27). It remains to be tested whether PARP1 expressing cells in the spleen show normal or higher levels of proliferation.

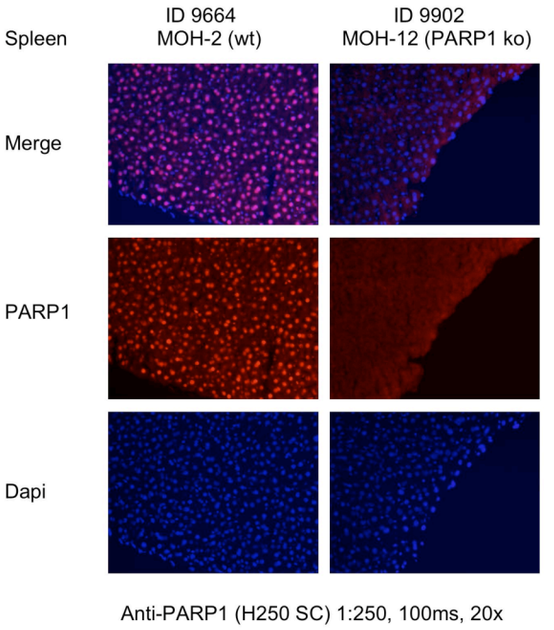


Figure 27: PARP1 is expressed in a subpopulation of mouse splenocytes. PARP1 was detected by immunofluorescence microscopy as described (Hapfelmeier et al., 2005) using cryosections stained with rabbit anti-PARP1 antibody H-250 (Santa Cruz), FITC-conjugated secondary antibody (Covance), and DAPI. Images were taken using an Olympus Mx51, NA 1.3 fluorescence microscope.

3 DISCUSSION

3.1 Summary of the results

We have investigated the molecular mechanism of PAR formation by PARP1 and the closely related PARP family members PARP2 and PARP3 [150]. PARP1 synthesized PAR more efficiently than PARP2. PARP3 on the other hand did not synthesize PAR, but showed mono-ADP-ribosylation activity. The generation of different chimera proteins revealed that the amino-terminal domains of PARP1, PARP2 and PARP3 cooperate tightly with their corresponding catalytic domains. The DNA-dependent interaction between the amino-terminal DNA-binding domain and the catalytic domain of PARP1 increased V_{max} and decreased the K_m for NAD^+. Furthermore, we found that glutamic acid residues in the auto-modification domain of PARP1 are not required for PAR formation. Instead, we identified individual lysine residues as acceptor sites for ADP-ribosylation. Two lysine residues within a nuclear localization signal were also identified in PARP2 as sites for auto-ADP-ribosylation [158, 159]. Interestingly, the acceptor sites for ADP-ribose in both PARP1 and PARP2 are also targets for acetylation by histone acetyltransferases (HATs), suggesting a functional cross-talk between ADP-ribosylation and acetylation [37, 158].

The amino-terminal core histone tails are especially rich in the basic amino acids lysine and arginine. We found that PARP1 specifically modifies the histone tails of H2A, H2B, H3, and H4. By applying an extensive deletion and mutation strategy for each tail, we determined the region important for modification, and identified individual putative acceptor sites for ADP-ribose. The identified lysine residues are known targets for histone acetylation and/or methylation, again suggesting an interesting cross-talk between PARylation and other lysine modifications at histone tails.

In collaboration with the group of Wolf-Dietrich Hardt (ETH Zurich), we investigated a possible role for PARP1 in *Salmonella enterica* serotype Typimurium-induced colitis (manuscript submitted). We demonstrated that PARP1 is expressed in the proliferative zone of cecum crypts and is associated with a faster pro-inflammatory host response to *Salmonella* infection. This accelerated response involved higher expression of pro-inflammatory genes and more severe inflammation with increased infiltration of immune cells. These findings link PARP1 to

Salmonella-induced pro-inflammatory gene expression and suggest an important role for PARP1 in controlling the host response to enteric *Salmonella* infection.

In collaboration with Isabella Toller and Anne Müller (both IMCR, UZH Zurich), we investigated the possible role of PARP1 in *Helicobacter pylori* induced gastric inflammation (manuscript in preparation). PARP1 was required for the efficient expression of several pro-inflammatory genes induced by *H. pylori* in AGS cells. Moreover, PAR formation was observed in gastric epithelial cells after infection with *H. pylori*. Finally, treatment of mice with PARP inhibitor PJ34 severely reduced *Helicobacter*-induced gastric inflammation (Isabella Toller, unpublished results). These data link PARP1 to *Helicobacter pylori* induced gastritis and gastric cancer.

PARP1 is a known transcriptional co-activator of NF-κB, a function, which may not require the enzymatic activity of PARP1 [108]. Using customized microarrays and quantitative RT-PCR, we identified a subset of NF-κB target genes, which was dependent on PARP1, but not on PARylation as tested with chemical PARP inhibitors, for efficient gene induction. PARP1 dependent genes have an altered nucleosome formation potential around the transcription start site. Moreover, at least some PARP1 dependent genes contain a recently identified consensus binding motif for the base excision repair protein and known PARP1 interactor XRCC1 in their promoter region.

Finally, in collaboration with the group of Martin Schwemmle (University of Freiburg, Germany), we investigated whether PARP1 was required for viral replication. Interestingly, semliki forest virus (SFV) was heavily dependent on PARP1 for successful replication in MLFs.

3.2 Lysine residues as acceptors for ADP-ribose

Biochemical studies in the 1980s had already aimed at identifying ADP-ribose acceptor sites in histones. Ogata et al. identified the glutamic acid residue at position 2 within the amino terminal tail of H2B as acceptor site for ADP-ribose when they incubated chromatin from rat liver with radioactive NAD^+ [151]. The same group also identified glutamic acid residues in the linker histone H1 as ADP-ribose acceptor sites [160]. Importantly, at that time no other PARP family member had been identified yet, and the tools to perform experiments in PARP1 negative systems, i.e. “loss-of-function” studies, were not yet available. Furthermore, the identified sites had never

been confirmed by mutational analysis. Thus, it may well be that another PARP family member or even another PARP1-unrelated NAD^+ consuming enzyme was responsible for the modification at the identified glutamates observed by Ogata and colleagues. In line with this notion, we observed no reduction in the modification of the H2B tail by recombinant purified PARP1 when we mutated the glutamate at position 2 of H2B to an alanine.

In a very recent publication, Tao et al. identified ADP-ribose acceptor sites in the auto-modification domain of a catalytic PARP1 mutant by LC-MS/MS [161]. The acidic amino acids D387, E488, and E491 were within peptides that were modified by PARP1 E988Q and were proposed to be primary or secondary acceptor sites for ADP-ribose. The use of relatively high concentrations of substrate NAD^+ (500μM) together with the relatively long incubation time (2h at room temperature) may, however, have resulted in the unspecific and/or non-enzymatic modification of the identified sites. Another major drawback of this study is the lack of evidence for ADP-ribosylation of these three amino acids by wild type PARP1. The detected acidic residues may only function as acceptors in the context of the tested PARP1 E988Q mutant, and lysine residues may represent the primary acceptor sites for ADP-ribose in wild type PARP1. Obviously, only the identification of ADP-ribose acceptor sites in the context of full-length wild type auto-modified PARP1, though certainly challenging, will help to resolve this issue.

The identification of lysine residues as acceptor sites for PARP1-mediated ADP-ribosylation has important implications for the reaction mechanism of PARylation. The initiation reaction, i.e. the attachment of the first ADP-ribose moiety onto an acceptor lysine, is not catalyzed by the amino acid E988 in PARP1, since an E988K mutant protein still possesses mono-ADP-ribosylation activity, while it is not able to catalyze PAR formation. Thus, E988 may be directly involved in chain elongation and possibly also branching, but not in the first step of PAR formation. A good candidate to catalyze the initiation reaction, however, is the amino acid K903, which is in very close distance to the substrate NAD^+. This lysine may stabilize a transition state after the cleavage of NAD^+ to nicotinamide and ADP-ribose and before the ADP-ribose is attached onto the acceptor lysine. Accordingly, a PARP1 K903A mutant would be predicted to lose the ability to catalyze mono-ADP-ribosylation. PARylation could consequently be mechanistically subdivided into the initiation reaction, which is catalyzed by K903 and transfers an ADP-ribose moiety

from NAD^+ to an acceptor lysine, and the elongation/branching reaction, which in contrast is catalyzed by E988 and transfers ADP-ribose moieties onto hydroxyl groups of ribose molecules of the growing PAR chain.

3.3 PARP1 and chromatin remodeling

Chromatin remodeling mechanisms can be divided into (1) post-translational histone modifications, (2) ATP-dependent remodeling and (3) incorporation of histone variants [162]. PARP1 has now been implicated in all three of these processes: PARP1 covalently modifies core histone tails (own results), regulates the ATPase ALC1 [67, 68] and FACT-mediated H2AX incorporation [69]. Histones are modified by a range of post-translational modifications including acetylation, methylation, phosphorylation, ubiquitylation, and ribosylation [163]. Histone hyperacetylation mediated by histone acetyl transferases (HATs) generally correlates with transcriptionally active genes, while histone hypoacetylation mediated by histone deacetylases (HDACs) correlates with silenced genes. H3K9, H3K14, H3K16, H4K9 and H4K16 are known target for acetylation [164]. Moreover, methylation of H3K9, H3K27 and H4K20 are generally associated with transcriptional silencing, whereas methylation of H3K4, H3K36 and H3K79 are associated with active chromatin [163]. Linker histone H1 stabilizes higher order chromatin structures and may function as global repressor of transcription. Interestingly, by using a ChIP-chip approach, the group of W.L. Kraus suggested that PARP1 and H1 bind reciprocally at chromatin and that PARP1 displaces H1 from the DNA [64].

We have identified in a first set of GST-histone tail deletion mutants the regions within the core histone tails that are targeted by PARP1 mediated PARylation. In a second set of GST histone tail mutants carrying single or multiple amino acid substitutions, we have identified the putative acceptor amino acids for ADP-ribose attachment. For histone H2A, the most prominent sites are lysines 13 and 15, two sites for which no other post-translational modification has been described before. For H2B, several lysine and/or arginine residues between lysine 27 and lysine 34 may be acceptors for ADP-ribose. Also for these residues, no other modification has been identified before. Lysines 23 and 27 were the most prominent ADP-ribose acceptor sites in H3, and for histone H4 we identified K16 and K20 as likely acceptor sites for PARylation. H3K23 is a target for ScSAS3 mediated acetylation, while H3K27 is a target for EZH2 mediated methylation in mammals, but not in budding and fission

yeast [52]. Hypermethylation of H3K27 in higher eukaryotes is associated with a silent chromatin state and transcriptional repression. In line, H3K27 methylation is thought to be involved in the maintenance of the inactive X chromosome [52]. Acetylation of H3K23, on the other hand, is less well investigated but may be a mark of open chromatin. H4K16 is acetylated by Tip60 and by ScSAS2 (SpMST2), whereas H4K20 is also methylated by Pr-SET7/8, SUV4 20H1, SUV4 20H2 and SpSet9 [52]. The acetylation of H4K16 is a fairly well described histone modification. It prevents the formation of the 30nm chromatin fiber and the generation of higher order chromatin structures [165, 166]. Deacetylation at H4K16 is achieved by the fission yeast class III deacetylase Sir2 and by the human Sir family member SirT2 [167]. SirT2 may thus have the ability to induce chromatin condensation *in vivo* by antagonizing H4K16 acetylation [52]. In contrast, methylation of H4K20 is strongly associated with silent heterochromatic regions and consequently with transcriptional repression. H4K20 methylation may be antagonized by the action of the JMJD2A lysine demethylase, which binds to H4K20me [168]. Interestingly, ionizing radiation induced DNA damage generates nuclear foci, which contain methylated H4K20 and p53BP1 to signal a G2/M arrest in order for the DNA to be repaired [169, 170]. As p53BP1 recruitment to these sites is dependent on H4K20 methylation, this modification has an important function in the DNA damage response apart from its role in transcription.

It will be important to confirm the identified putative sites of PARP1 mediated histone ADP-ribosylation by mass spectrometry *in vitro* and eventually also *in vivo*, before the functional consequences of histone ADP-ribosylation can be addressed in a truly rational manner. It is, however, intriguing to already speculate about the potential cross-talks with other post-translational modifications at the same sites, at sites nearby, or even at sites further apart. PARylation and other histone modifications may be either mutually exclusive or coincide within certain chromatin domains. The tools to address this point and study global histone PARylation at specific amino acids are not yet available, but antibodies raised against single ADP-ribosylated residues within histone tails will hopefully allow for ChIP-chip analysis of histone PARylation. In parallel, the mechanistic analysis of the cross-talk between PARylation and histone acetylation by histone acetyltransferases or histone methylation by methyltransferases will provide important insights into the complex regulation of chromatin function by these modifications. Moreover, to investigate the

interplay between PARP1 and other chromatin-associated NAD^+-consuming enzymes, such as the Sir family members, will most probably reveal fascinating links between chromatin modifications and cellular metabolism. Chromatin structure may be the platform to integrate the great variety of internal and external signals and translate them into an appropriate response in terms of DNA replication, repair or transcription, always in the light of cellular energy status. PARP1, with its important roles in transcription and repair, and as a cellular sensor of energy levels, may be a central component of this nuclear integration hub. It will be interesting to study how PARP1 and SirT2 cooperate to allow for H4K16 deacetylation and subsequent ADP-ribosylation, for example, and how the same two enzymes may concurrently compete for the same substrate.

Several post-translational histone modifications are recognized by specialized protein domains. Methylated lysines, for instance, are bound by chromo-like domains of the Royal family (chromo, tudor, MBT) and non-related PHD domains [52]. Acetylation marks, in contrast, are read by bromodomains, and phosphorylation is recognized by a domain within 14-3-3 proteins. PARP1 generated PAR was already described to be recognized by at least three different protein motifs, by a basic PAR binding motif, by PAR binding zinc fingers, and by macrodomains [62, 63, 72]. Whether proteins, which harbor these PAR sensing domains, would also recruit to PARylated histone tails has not been addressed experimentally yet. It is tempting to suggest, however, that macrodomain containing histone variants like H2A1.1, for example, would bind to PARylated core histone tails to mediate chromatin composition and function. Strikingly, all regions within the core histone tails, which are targeted by PARP1 mediated ADP-ribosylation, are close to the approximate sites where the tails exit the DNA superhelical gyres to the exterior of the nucleosome [152, 153]. This indicates, at least in principle, that histone tail PARylation and its recognition by PAR binding domains regulate nucleosome structure and/or the generation of higher order chromatin structures. From both charge and structure of PAR one would expect that histone tail PARylation can decompact heterochromatin and/or prevent free histones from being integrated into nucleosomes. Both processes may be especially important during replication, where heterochromatin needs to be dissolved and new nucleosomes have to be formed.

Finally, most histone modifications are reversible and also PARylation is a transient and in general reversible process. As important as the analysis of PARP1

functions for chromatin structure modulation may thus be the analysis of PAR degrading enzymes, such as PARG and ADP-ribosyl protein lyase, and their contribution to the regulation of histone tail PARylation. Especially, the ADP-ribosyl protein lyase is only poorly described and it will be important to study whether histone tail PARylation is indeed fully reversible, or whether the histone proximal ADP-ribose unit remains attached to the tail as a stabile mark. A stabile histone tail mark, which could only be removed by replacing the affected histone with a non-marked histone, could be part of a chromatin memory and function as an epigenetic mechanism.

In summary, PARP1 mediated histone tail PARylation occurs as post-translational modification at distinct lysine residues. The PARylation mark can be read by special PAR binding domains and probably has important cellular implications for processes such as histone dimerization or multimerization, histone shuttling, nucleosome formation or regulation of higher order chromatin structure. Since ADP-ribosylation of lysines not only neutralizes the positive charge of the amino acid side chain (as does lysine acetylation), but instead reverses it into a negative charge, the effects of lysine ADP-ribosylation can be assumed to be even more drastic than acetylation and may be especially required not only to prevent heterochromatin formation, but to unfold dense heterochromatic genomic regions. In contrast to mono-ADP-ribosylation of lysines, PARylation would even add a complex, bulky and highly anionic molecule to histone tail lysines. Accordingly, the effects of PARylation for unfolding heterochromatin can be expected to be much stronger than the attachment of a single ADP-ribose unit onto a histone tail lysine.

3.4 PARP1 and NF-κB dependent transcription

The role of PARP1 for NF-κB-dependent gene expression was analyzed in different systems. Interestingly, one of the genes, which was most robustly down-regulated after an inflammatory stimulus in cells and tissues lacking PARP1, was the inhibitor of NF-κB IκBε. Messenger RNA levels of the IκBε gene after LPS treatment were considerably lower in Raw264.7 macrophages depleted for PARP1 and in peritoneal macrophages isolated from PARP1 knockout mice as compared to PARP1 proficient control cells. IκBε transcripts were also lower in the gastric epithelium of PARP1 knockout mice after *in vivo* infection with *Helicobacter felis* than in tissue isolated

from infected wild type mice. IκBε is one of several inhibitory proteins for NF-κB dependent transcription. Recently, it was suggested that IκBε is especially important for a negative feedback loop that increases the cell-to-cell heterogeneity of the NF-κB response, which originates from intrinsic, stochastic transcriptional variability [171]. To determine the physiological importance of oscillatory gene expression and cell-to-cell-heterogeneity in the inflammatory immune response is a challenge of future research and it will be interesting to elucidate the role of PARP1 in these still poorly understood phenomena. It seems obvious, however, that the misregulation of an NF-κB feedback loop in cells lacking PARP1 may also indirectly influence the expression levels of other NF-κB target genes.

From several customized microaray experiments, PARP1 and stimulus dependent genes can be summarized to mainly fall into four different categories: (1) genes upregulated after stimulation in wild type but not in PARP1 deficient cells ("co-activator"), (2) genes downregulated after stimulation in wild type but not in PARP1 deficient cells ("co-repressor"), (3) genes upregulated after stimulation in PARP1 deficient but not in wild type cells ("co-repressor"), (4) genes downregulated after stimulation in PARP1 deficient but not in wild tpye cells ("co-activator").

It is more and more appreciated that transcription factors often work in concert with a multitude of co-factors, which regulate and fine-tune gene expression. Moreover, different transcription factors may cooperate on a gene promotor for complete activation of transcription. Indeed, many pro-inflammatory genes are responsive to NF-κB and the interferon responsive transcription factor STAT1 and contain binding sites for both transcription factors in their promoters [172]. NF-κB and STAT1 can thus have distinct as well as synergistic functions on gene expression. Of note, STAT1 transcript levels are regulated by NF-κB, as are other transcription factors involved in the immune response, for example IRFs and SRF [173].

3.5 *Helicobacter pylori* and DNA damage

The host response to *Helicobacter pylori* is still largely undefined and a matter of current investigations. It has been noted, however, that *Helicobacter pylori* can cause DNA damage in gastric epithelial cells [174]. DNA fragmentation in AGS cells incubated with *Helicobacter pylori* extract correlated with increased levels of reactive oxygen species and reduced levels of glutathione. Moreover, also PAR formation was

observed [174]. Apart from this observation, the role of PARP1 for *Helicobacter pylori* induced host cell responses has not been studied extensively. Of note, *Helicobacter pylori* leads to the expression of NF-κB target genes via a mechanism involving the *H. pylori* virulence factor CagA and the host protein transforming growth factor-β-activated kinase 1 (TAK1), which activates IKK to phosphorylate IκBa [175]. We observed that *H. pylori* induced NF-κB target genes in AGS cells and that some of these genes required PARP1 for efficient expression. Interestingly, *H. pylori* induced activation-induced deaminase (AID) independent of PARP1. AID was very recently described to induce DNA double strand breaks in non-Ig genes throughout the genome [176] and it is therefore tempting to suggest that *H. pylori* induced AID expression results in DNA double strand break formation in AGS cells, which in turn activate PARP1 and hence would be responsible for the observed PARP1-dependent PAR formation. Knockdown of AID in AGS cells would consequently be expected to reduce *H. pylori* induced DNA damage and PAR formation.

3.6 A stochastic model for PARP1 dependent gene expression

Transcription is a per se stochastic process and is preceded by multiple cellular events including chromatin decondensation, nucleosome remodeling, histone modifications, binding of transcriptional activators and co-activators to enhancers and promoters, and recruitment of the basal transcription machinery to the core promoter [177].

PARP1 is involved in transcription of genes under basal, unstimulated conditions but seems to be even more important for transcription of inducible genes after stimulation. PARP1 dependent inducible gene expression may predominantly be independent of PARP1 catalytic activity. Under certain conditions, however, gene expression may also depend on PAR formation. Furthermore, only subsets of genes analyzed in microarray studies show PARP1 dependency. PARP1 can enhance the expression of some ("co-activator") and repress the expression of other genes ("co-repressor"). Depending on the type of stimulus, stimulus strength, timescale, cell cycle stage and cell type or tissue, different subsets of genes may be PARP1 dependent. Furthermore, up to now little is known about the possible roles of other PARP family members for transcription and it will be interesting to investigate a putative interplay between PARPs and PARP-like enzymes during gene expression.

Whereas the enzymatic activity of PARP1 may not be required for pro-inflammatory gene expression in the absence of DNA damage, activities of other PARP family members, e.g. the DNA-associated tankyrases, may well have a relevant function for gene expression under certain conditions. In line with this notion, it was recently shown that tankyrase inhibition suppresses β-catenin target gene expression [178]. This finding supports the idea, that PAR formation by tankyrases or other PARP family members may have so far undiscovered and undervalued functions for processes like transcription, replication or repair.

PARP1 may be especially required for efficient gene expression under conditions where one or more component of the pre-initiation complex becomes limiting. These components may be inducible or non-inducible transcription factors, transcriptional co-activators or co-repressors, chromatin modifying or remodeling proteins or any other chromatin associated process that influences formation of the pre-initiation complex and the onset of transcription. Such limiting conditions may arise more likely under stimulated conditions, where gene expression is enhanced manifold, as compared to basal conditions with constitutive and moderate gene expression. Limiting conditions will not affect all genes, but instead will stochastically affect only certain loci on the genome and thus only a subset of genes. In other words, where, i.e. at which genomic locus, a limitation will occur, will largely have random character and differ from cell to cell. Especially in analyses of large cell populations in culture or in tissue, these limiting conditions at individual genomic loci in individual cells would generally disappear in the noise of the majority of cells which do not have a limitation at the same locus.

The notion that PARP1 acts as a safeguard for efficient gene expression under conditions where one or more factors involved in transcription initiation become limiting, is consistent with a variety of experimental findings: (1) PARP1 is a highly abundant nuclear protein with high but sequence unspecific affinity for DNA and it has been estimated that one PARP1 molecule is present every 500 bases along the 3 billion bases of the human genome. This high global abundance would allow PARP1 to support the transcription machinery wherever limiting conditions arise; (2) up to now we have failed to clearly define the characteristics of PARP1 dependent or independent genes. We do not know exactly what makes a gene PARP1 dependent nor do we have a molecular understanding of why certain genes are PARP1 dependent under one condition but are PARP1 independent under another condition.

A stochastic model for PARP1 dependent gene expression under limiting conditions would fully explain these observations; (3) PARP1 can both up-regulate some and down-regulate other genes. In fact, the regulation of PARP1 dependent genes is complex and probably includes all theoretically possible varieties. A stochastic model explains why PARP1 has both co-activator and co-repressor functions; (4) PARP1 dependent gene expression in animal models is observed mostly under acute and strong stimulation, e.g. ischemia-reperfusion models or LPS-induced septic shock. Under physiological conditions, where limiting conditions are unlikely to occur *in vivo*, PARP1 may be nonessential and indeed PARP1 knockout mice show no obvious phenotype under normal conditions.

Last, it may be worthwhile to speculate whether a stochastic model for PARP1 dependent gene expression can also be applied to the role of PARP1 in the DNA damage response. Also here, PARP1 may be the safeguard that comes into play under limiting conditions. If a certain DNA damage response pathway is overloaded, PARP1 may be required to faithfully and efficiently launch the repair. Consistent with this notion, many publications have described PARP1 as being involved in most known DNA repair pathways; the true significance of PARP1 for the repair may, however, only show under conditions where a PARP1 independent repair pathway is already compromised. In line, a DNA safeguard function of PARP1 would explain the immense potential of PARP inhibitors in cancer treatment of tumors with defects in the DNA repair machinery.

In conclusion, PARP1 may be regarded as safeguard for transcription and DNA repair under otherwise insufficient, limiting conditions. From an evolutionary perspective, such limiting conditions may have arisen once genomes became bigger and, in fact, the only eukaryotes, which do not encode a PARP1 homologue, are yeasts. These unicellular organisms have small genomes (in the order of 10^6-10^7 bp) as compared to other eukaryotes (generally $>10^8$ bp) and therefore may very rarely experience limiting conditions.

3.7 Perspectives

The identification of lysine residues instead of glutamates as acceptor sites for ADP-ribose has challenged a dogma in the field of PARP research. Although technically challenging, it will be important to substantiate this finding by mass spectrometric analysis of ADP-ribosylated peptides *in vitro*. Eventually, the combined information

from mutational studies and mass spectrometry will hopefully yield a complete picture of the primary acceptor sites for covalent ADP-ribose attachment by PARP1. The identification of ADP-ribose acceptor sites will increase our understanding of both the auto-modification of PARP1 (and of other PARP family members) and the modification of acceptor proteins (e.g. histones). Moreover, it should be possible to generate specific antibodies against single ADP-ribosylated lysine residues, which will provide a great tool to study the many functions of PAR formation for chromatin remodeling, transcription and DNA repair in living cells and animal tissue. Certainly, this would reveal interesting and so far unforeseen insights into PARP1 functions.

We still do not have a completely comprehensive understanding of the role of PARP1 for transcription. Difficulties to address certain individual aspects of PARP1 function may arise due to the many and in part overlapping functions PARP1 has in the context of chromatin. PARP1 changes chromosome composition and compaction, influences both basal and inducible gene expression, functions as co-activator and co-repressor and may have different roles depending on cell type and condition. Moreover, some experimental approaches may suffer from the partial redundancy of PARP1 and PARP2 functions, and from the lack of truly specific PARP inhibitors.

Importantly, PARP1 depletion or inhibition of PAR formation is beneficial *in vivo* in a variety of inflammation-associated diseases, such as *Salmonella* induced colitis (manuscript submitted) or *Helicobacter pylori* induced gastritis and gastric cancer (manuscript in preparation). Our understanding of the molecular mechanisms behind these beneficial effects, however, is only limited. Conditional knockout mice, in which both PARP1 and PARP2 can be simultaneously eliminated from a certain tissue, would provide a great tool to study the individual contribution of these two enzymes for different diseases. Also, better PARP inhibitors to target specifically PARP1, PARP2, or other PARP family members with negligible off-target effects would provide a means to dissect their individual functions. New technologies together with the careful investigation of observed putative PARP effects and how they are linked to PARP1, PARP2, other PARPs, mARTs or NAD^+-consuming enzymes will further expand our understanding of this exciting family of enzymes and their contribution to health and disease.

4 ABBREVIATIONS

53BP1	p53 binding protein 1
AD	auto-modification domain
ADP	adenosine diphosphate
AID	activation-induced deaminase
AIF	apoptosis inducing factor
ALC1	amplified in liver cancer 1
AP-1	activator protein 1
ATM	ataxia telangiectasia mutated
ATP	adenosine triphosphate
Bp	base pair
BRCA1	breast cancer type 1 susceptibility protein
BRCT	BRCA1 C-terminus
CAT	catalytic domain
ChIP	chromatin immunoprecipitation
CTCF	C_2H_2-type zinc fingers
DBD	DNA binding domain
DNA	desoxyribonucleic acid
DNMT1	DNA methyltransferase 1
FACT	facilitates chromatin transcription
FOXO1	forkhead box O transcription factor
FPV	feline panleukopenia virus
GST	glutathione S-transferase
HA	hemagglutinin
HAT	histone acetyltransferase
His	histidine
HP1	heterochromatin protein 1
IMPGEs	immortalized primary gastric epithelial cells
kDa	kilo dalton
LC-MS	liquid chromatography mass spectrometry
mART	mono-ADP-ribosyltransferase
MLF	mouse lung fibroblasts

MRE11	meiotic recombination 11 homolog
mRNA	messenger ribonucleic acid
NaCl	sodium chloride
NAD	nicotinamide adenine dinucleotide
NBS1	nijmegen breakage syndrome protein 1
NF-κB	nuclear factor-kappaB
NFAT	nuclear factor of activated T cells
Ni	nickel
NLS	nuclear localization signal
NRF1	nuclear respiratory factor 1
Oct-1	octamer-binding transcription factor 1
PAR	poly(ADP-ribose)
PARG	poly(ADP-ribose) glycohydrolase
PARP	poly(ADP-ribose) polymerase
PARylation	poly(ADP-ribosyl)ation
PTEN	phosphatase and tensin homolog
RHD	Rel homology domain
ROS	reactive oxygen species
RT-PCR	reverse transcription polymerase chain reaction
SFV	semliki forest virus
SIRT	Sir2-type
TEF-1	transcription enhancer factor 1
TopBP1	topoisomerase binding protein 1
TSS	transcription start site
WGR	tryptophane, glycine, arginine domain
XRCC1	x-ray repair cross-complementing protein 1
YY-1	ying-yang-1

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